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ISOLATED DIMERIC FIBROBLAST ACTIVATION PROTEIN ALPHAS, AND USES THEREOF

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(54) Title: ISOLATED DIMERIC FIBROBLAST ACTIVATION PROTEIN ALPHA, AND USES THEREOF			
(57) Abstract <p>The invention involves dimeric forms of the protein known as fibroblast activation protein alpha, or "FAP<math>\alpha</math>" and its uses.</p>			
<p style="text-align: center;">293-FAP 293</p> <p style="text-align: right;">— 205 kD — 116 kD — 97.4 kD — 66 kD</p>			

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ISOLATED DIMERIC FIBROBLAST ACTIVATION  
PROTEIN ALPHA, AND USES THEREOF

RELATED APPLICATION

5 This application is a continuation-in-part of Serial No. 08/230,491, filed April 20, 1994, now pending and incorporated by reference.

FIELD OF THE INVENTION

10 This invention relates to certain molecules associated with cancer tissues and reactive tumor stromal cells. More particularly, it relates to fibroblast activation protein alpha ("FAP $\alpha$ " hereafter) molecules. A monomeric form of the molecule has previously been identified immunochemically, but nucleic acid molecules coding for it had not been isolated or cloned nor have dimers been identified. These, *inter alia*, are features of the invention. The monomeric protein has a molecular weight of from about 88 to about 95 kilodaltons as determined by SDS-PAGE of boiled samples. The dimer has a molecular weight of about 170 kilodaltons as determined by SDS-PAGE of unboiled samples. FAP $\alpha$  is characterized by a number of features and properties which are shared by and characteristic of membrane bound enzymes, suggesting very strongly that it, too, is a membrane bound enzyme. The nucleic acid molecules, which are a key part of the invention, are useful both as probes for cells expressing FAP $\alpha$ , and as starting materials for recombinant production of the protein. The FAP $\alpha$  protein can then be used to produce monoclonal antibodies specific for the protein and are thus useful diagnostic agents themselves. They also have additional uses, including uses related to enzymatic functions, as described herein.

BACKGROUND AND PRIOR ART

30 The invasive growth of epithelial cancers is associated with characteristic cellular and molecular changes in the supporting stroma. For example, epithelial cancers induce the formation of tumor blood vessels, the recruitment of reactive tumor stromal fibroblasts, lymphoid and phagocytic

infiltrates, the release of peptide mediators and proteolytic enzymes, and the production of an altered extracellular matrix (ECM). See, e.g., Folkman, *Adv. Cancer Res.* 43: 175-203 (1985); Basset et al., *Nature* 348: 699-704 (1990); Denekamp et al., *Cancer Metastasis Rev.* 9: 267-282 (1990); Cullen et al., *Cancer Res.* 51: 4978-4985 (1991); Dvorak et al., *Cancer Cells* 3: 77-85 (1991); Liotta et al., *Cancer Res.* 51: 5054s-5059s (1991); Garin-Chesa et al., *J. Histochem. Cytochem.* 37: 1767-1776 (1989). A highly consistent molecular trait of the stroma in several common histologic types of epithelial cancers is induction of the fibroblast activation protein (FAP $\alpha$ ), a cell surface glycoprotein with an observed M<sub>r</sub> of 95,000 originally discovered with a monoclonal antibody, mAb F19, raised against proliferating cultured fibroblasts. See Rettig et al., *Cancer Res.* 46: 6406-6412 (1986); Rettig et al., *Proc. Natl. Acad. Sci. USA* 85: 3110-3114 (1988); Garin-Chesa et al., *Proc. Natl. Acad. USA* 87: 7235-7239 (1990); Rettig et al., *Cancer Res.* 53: 3327-3335 (1993). Each of these four papers is incorporated by reference in its entirety.

Immunohistochemical studies such as those cited supra have shown that FAP $\alpha$  is transiently expressed in certain normal fetal mesenchymal tissues but that normal adult tissues are generally FAP $\alpha$ <sup>-</sup>. Similarly, malignant epithelial, neural and hematopoietic cells are generally FAP $\alpha$ <sup>-</sup>. However, most of the common types of epithelial cancers, including >90% of breast, lung, skin, pancreas, and colorectal carcinomas, contain abundant FAP $\alpha$ <sup>+</sup> reactive stromal fibroblasts. Garin-Chesa et al., *Proc. Natl. Acad. Sci. USA* 87: 7235-7239 (1990). The FAP $\alpha$ <sup>+</sup> tumor stromal fibroblasts almost invariably accompany tumor blood vessels, forming a distinct cellular compartment interposed between the tumor capillary endothelium and the basal aspect of malignant epithelial cell clusters. While FAP $\alpha$ <sup>+</sup> stromal fibroblasts are found in both primary and metastatic carcinomas, benign and premalignant epithelial lesions, such as fibroadenomas of the breast and colorectal adenomas only rarely contain FAP $\alpha$ <sup>+</sup> stromal cells. In contrast

to the stroma-specific localization of FAP $\alpha$  in epithelial neoplasms, FAP $\alpha$  is expressed in the malignant cells of a large proportion of bone and soft tissue sarcomas. (Rettig et al., Proc. Natl. Acad. Sci. USA 85: 3110-3114 (1988)). Finally, 5 FAP $\alpha$  fibroblasts have been detected in the granulation tissue of healing wounds (Garin-Chesa et al., *supra*). Based on the restricted distribution pattern of FAP $\alpha$  in normal tissues and its uniform expression in the supporting stroma of many epithelial cancers, clinical trials with  $^{131}\text{I}$ -labeled mAb F19 10 have been initiated in patients with metastatic colon cancer (Welt et al., Proc. Am. Assoc. Cancer Res. 33: 319 (1992); Welt et al. J. Clin. Oncol. 12: 1561-1571 (1994)) to explore the concept of "tumor stromal targeting" for immunodetection and immunotherapy of epithelial cancers.

15 Rettig et al., Int. J. Cancer 58: 385-392 (1994), incorporated by reference, discusses the FAP $\alpha$  molecule and its features. Rettig et al postulate that FAP $\alpha$  is found in high molecular weight complexes in excess of 400 kilodaltons, but do not discuss the possibility of dimeric molecules, nor does 20 the paper elaborate on the specific enzymatic properties of the molecule.

25 The induction of FAP $\alpha$  fibroblasts at times and sites of tissue remodeling during fetal development, tissue repair, and carcinogenesis is consistent with a fundamental role for this molecule in normal fibroblast physiology. Thus, it is of interest and value to isolate and to clone nucleic acid molecules which code for this molecule. This is one aspect of the invention, which is described in detail together with other features of the invention, in the disclosure which 30 follows. Further aspects of the invention include the dimeric FAP $\alpha$  molecules, and the exploitation of the properties of these molecules. These features are also elaborated upon hereafter.

**BRIEF DESCRIPTION OF THE FIGURES**

35 Figure 1 compares the deduced amino acid sequence for FAP $\alpha$ , and the known sequence of CD26. The alignment has been optimized.

Figures 2A-2H, inclusive, display immunohistochemical detection of FAP $\alpha$  and CD26 in various tissues. In figures 2A and 2B, breast cancer is studied, for FAP $\alpha$  (figure 2A), and CD26 (figure 2B). In figures 2C and 2D, malignant fibrous histiocytoma is studied, for FAP $\alpha$  (figure 2C), and CD26 (figure 2D). Dermal scar tissue is examined in figures 2E (FAP $\alpha$ ), and 2F (CD26). Renal cell carcinoma is studied in figure 2G (FAP $\alpha$ ), and 2H (CD26).

Figure 3 presents some of the data generated in experiments which showed that FAP $\alpha$  had extracellular matrix (ECM) protein degrading activity. When zymographic detection of gelatin degrading extracts of 293-FAP was carried out, the active substance was found to have a molecular weight of about 170 kD, via SDS-PAGE, using unboiled samples to preserve enzyme activity.

DETAILED DESCRIPTION OF PREFERRED EMBODIMENTS

Example 1

Fibroblast cell line WI-38 had been observed, previously, to react with mAb F19 (Rettig et al., Canc. Res. 46: 6406-6412 (1986); Rettig et al., Proc. Natl. Acad. USA 85: 3110-3114 (1988); Garin-Chesa et al., Proc. Natl. Acad. Sci. USA 87: 7235-7239 (1990); Rettig et al., Canc. Res. 53: 3327-3335 (1993)). It was used in the experiments which follow.

A cDNA library was prepared from WI-38, using well known techniques and commercially available materials. Specifically, the library was constructed in expression vector pCDNAI, using the Fast Track mRNA isolation kit, and Librarian cDNA phagemid system. Once the library was prepared, the vectors were electroporated into cell line *E. coli* MC 1061/P3. The pCDNAI expression vector contains an antibiotic resistance gene, so the *E. coli* were selected via antibiotic resistance. The colonies which were resistant were then used in further experiments. The plasmid DNA from the colonies was obtained via alkaline lysis and purification on CsCl, in accordance with Sambrook et al, Molecular Cloning: A Laboratory Manual (Cold Spring Harbor Lab, Cold Spring Harbor, N.Y. 2d Ed. 1989). The technique is well known to the art, but is

incorporated by reference herein.

Once the plasmid DNA was isolated, it was used to transfect COS-1 cells, which were then cultured for forty-eight hours, after which these were tested with antibody coated dishes. The mAbs used included F19, as described by Rettig et al., (1986), supra, which is incorporated by reference in its entirety. As COS-1 cells are normally FAP $\alpha$ , any positive results indicated the presence of the coding sequence. The immunoselection protocol was that of Aruffo et al., Proc. Natl. Acad. Sci USA 84: 3365-3369 (1987), incorporated by reference herein.

Plasmid DNA from positive clones was recovered, in accordance with Hirt, J. Mol. Biol. 26: 365-369 (1967), reintroduced into *E. coli* MC 1061/P3, and reselected in COS-1 cells.

The protocol presented herein was followed for four rounds. After this, the plasmid DNA of 50 isolated bacterial colonies was purified, using the Qiagen plasmid kit. Of the colonies, 27 clones were found to contain identical 2.8 kb inserts, as determined by EcoRI restriction enzyme mapping. Several of these were found to contain FAP $\alpha$ -specific cDNA as determined by transient expression in COS-1 cells and direct immunofluorescence staining with mAb F19. One of these clones, i.e., "pFAP.38" was selected for further study, as elaborated upon infra.

Example 2

Once pFAP.38 had been identified, it was tested together with a vector coding for known cell surface marker CD26 ("pCD26"), as well as with control vector pCDNA I.

In these experiments, COS-1 cells were transfected with one of pFAP.38, pCD26, or pCDNAI. After forty-eight hours, the transfectants were tested, using the well known MHA rosetting assay for cell surface antigen expression. In these experiments, mAb F19, which is FAP $\alpha$  specific, was used, together with mAb EF-1, which is CD26 specific. Also used were four other FAP $\alpha$  specific mAbs, i.e., FB23, FB52, FB58 and C48. Also tested were two cancer cell lines, which are known

to react with mAb F19 (SW872 liposarcoma), or EF-1 (SK-OV6 ovarian cancer). The results are set forth in Table 1, which follows.

5      Table 1. Cell surface expression of multiple FAP $\alpha$  epitopes and CD26 in human cells and COS-1 cell transfectants

Cell surface antigen expression							
10	Target cell	F19	FB23	FB52	FB58	C48	EF-1
15	<u>Human cells</u>						
20	SW872 liposarcoma	>95%	>95%	>95%	>95%	>95%	-
25	SK-OV6 ovarian cancer	-	-	-	-	-	>95%
<u>COS-1 transfectants</u>							
30	COS-pCDNAI control	-	-	-	-	-	-
35	COS-pFA P 38	40%	30%	40%	20%	20%	-
	COS-pCD26	-	-	-	-	-	40%

35

Example 3

Immunoprecipitation studies were then carried out to identify the antigen being targeted by the antibodies.

40

Cells were metabolically labelled with Trans  $^{35}$ S-label, (ICN), extracted with lysis buffer (0.01 M Tris-HCl/0.15 M NaCl/0.01 M MgCl<sub>2</sub>/0.5% Nonidet P-40/aprotinin (20 ug/ml)/2 mM phenylmethyl-sulfonyl fluoride), and then immunoprecipitated. The protocols used are all well known, as will be seen by reference to Rettig et al., Canc. Res. 53: 3327-3335 (1993); and Fellinger et al., Canc. Res. 51: 336-340 (1991), the disclosures of which are all incorporated by reference in

45

their entirety. Precipitating mAbs were negative control mouse Ig, mAb F19, or EF-1. Control tests were carried out with mock transfected COS-1 cells. Following immunoprecipitation, the immunoprecipitates were boiled in extraction buffer and separated by NaDODSO<sub>4</sub>/PAGE, under reducing conditions. In some experiments, an additional test was carried out to determine whether or not the immunoprecipitated material was glycosylated. In these experiments, cell extracts were fractionated with Con A-SEPHAROSE prior to immunoprecipitation. Following immunoprecipitation, but prior to fractionation on NaDODSO<sub>4</sub>/PAGE, these precipitates were digested with N-Glycanase.

The results showed that, in COS-1 cells, pFAP.38 directs expression of an 88 kd protein species (as determined via SDS-PAGE), which is slightly smaller than the 95 kd FAP $\alpha$  species produced by SW872, or cultured fibroblasts. Digestion with N-Glycanase produced peptides of comparable size (i.e., 74 kd versus 75 kd), showing that the glycosylation of the FAP $\alpha$  protein in COS-1 cells is different than in the human cell lines.

Example 4

Classic Northern blot analysis was then carried out, using the mRNA from FAP $\alpha$  fibroblast cell lines WI-38 and GM 05389, and FAP $\alpha$ -ovarian cancer cell line SK-OV6. Using the procedures of Sambrook et al., *supra*, five micrograms of mRNA from each cell line were tested. The probes used were <sup>32</sup>P labelled, and were prepared from a 2.3 kb ECO I fragment of pFAP.38, a 2.4 kb Hind III fragment of CD26, and a 1.8 kb BamHI fragment of  $\gamma$ -actin cDNA. These fragments had been purified from 1% agarose gels.

The extracts of FAP $\alpha$  fibroblast strains showed a 2.8 kb FAP mRNA species, but extracts of SK-OV6 do not. A  $\gamma$ -actin mRNA species (1.8 kb), was observed in all species.

Example 5

The cDNA identified as coding for FAP $\alpha$  was subjected to more detailed analysis, starting with sequencing. The classic

Sanger methodology, as set forth in Proc. Natl. Acad. Sci. USA 74: 5463-5467 (1977), was used to sequence both strands of the cDNA. Once this was secured, an amino acid sequence was deduced therefrom. This information is presented in SEQ ID NO: 1. The sequence was then compared to the known amino acid sequence of CD26 (Morimoto et al., J. Immunol. 143: 3430-3437 (1989)). Figure 1 presents the comparison, using optimized sequence alignment. Any gaps in the comparison are indicated by asterisks, while identical amino acids are shown by dashes in the CD26 sequence. A hydrophobic, putative transmembrane sequence is double underlined, while potential N-glycosylation sites are single underlined.

The sequence analysis shows a 2812 base pair insert, wherein 2277 base pairs constitute the open reading frame. This ORF extends from start codon ATG at nucleotide 209, to stop codon TAA at 2486.

The deduced polypeptide is 760 amino acids long, and has a molecular weight of 87,832. In contrast, N-Glycanase digested, immunopurified FAP $\alpha$  was reported to have an estimated  $M_r$  of 75,000 on NaDODSO<sub>4</sub>/PAGE (Rettig et al., Canc. Res. 53: 3327-3335 (1993)). A GenBank data base search was carried out. The most closely related genes found were those encoding dipeptidyl peptidase IV homologues (DPPIV; EC 3.4.14.5), with human DPPIV (also known as T-cell activation antigen CD26), showing 61% nucleotide sequence identity, and 48% amino acid sequence identity.

The second set of related genes are human, rat, and bovine homologues of DPPX, a gene of unknown function widely expressed in brain and other normal tissues. The predicted human DPPX gene product shows about 30% amino acid sequence identity with FAP $\alpha$  and CD26. The FAP $\alpha$  molecule exhibits structural features typical of type II integral membrane proteins, including a large COOH-terminal extracellular domain, a hydrophobic transmembrane segment, and a short cytoplasmic tail. The putative extracellular domain contains five potential N-glycosylation sites, eleven cysteine residues (eight of which are conserved between FAP $\alpha$  and CD26), and

three segments corresponding to highly conserved catalytic domains characteristic of serine proteases, such as DPPIV. These conserved sequences are presented in Table 2, which follows. Comparisons to DPPIV and DPPX were made via Morimoto et al., *supra*; Wada et al., Proc. Natl. Acad. Sci. USA 89: 197-201 (1992); Yokotani et al., Human Mol. Genet. 2: 1037-1039 (1993).

Example 6

An additional set of experiments were carried out to determine whether FAP $\alpha$  related sequences are present in non-human species. To do so, human, mouse, and Chinese hamster genomic DNA was digested using restriction enzymes, and tested, via Southern blotting, using the 2.3 kb fragment, labelled with  $^{32}$ P, described supra. Hybridization was carried out using stringent washing conditions (0.1 x SSC, 0.1% NaDODSO<sub>4</sub>, 68°C). Cross-hybridization was readily observed with both the mouse and hamster DNA, suggesting the existence of highly conserved FAP $\alpha$  homologues. In control experiments using the CD26 cDNA fragment described supra, no evidence of cross hybridization was observed.

Example 7

The CD26 molecule shares a number of biochemical and serological properties with FAP $\beta$ , which is a previously described, FAP $\alpha$  associated molecule having a molecular weight of 105 kd, and is found on cultured fibroblasts and melanocytes (Rettig et al., Canc. Res. 53: 3327-3335 (1993)). Cotransfection experiments were carried out to determine whether FAP $\beta$  is a CD26 gene product. To test this, the same protocols were used which were used for transfection with pFAP.38 or pCD26, as described supra, but using the two vectors. The results presented supra showed that cotransfection efficiency was about 40% for each vector, so about 10-20% of cell should be cotransfected.

Following cotransfection, the COS-1 cells were Trans  $^{35}$ S-labeled, as described supra, then lysed, also as described supra.

The resulting cell extracts were separated on Con A

5           SEPHAROSE, and the antigen (FAP $\alpha$  and/or CD26) were recovered in the Con A-bound fraction. The bound fraction was eluted with 0.25 M  $\alpha$ -D-mannopyranoside. Immunoprecipitation was then carried out, as described supra, and the precipitates were separated on NaDdSO<sub>4</sub>/PAGE, also as discussed supra.

10           Those cells transfected only with pFAP.38 produced FAP $\alpha$ , but not FAP $\beta$  (determined from mAb F19 immunoprecipitates). They also produce no CD26 antigen (tested with EF-1). Those cells transfected with pCD26 alone produce CD26 but no FAP $\alpha$ .  
15           Cotransfectants produce CD26 and FAP $\alpha$ /FAP $\beta$  heteromers, as determined in the mAb F19 precipitates. This result provides direct evidence that FAP $\beta$  is a CD26 gene product.

Example 8

15           It has been observed previously that some cultured human cell types coexpress FAP $\alpha$  and CD26, and show FAP $\alpha$ /CD26 heteromer formation. In vivo distribution patterns of FAP $\alpha$  and CD26, however, as determined in previous immunohistochemical studies, appeared to be non-overlapping. (See Rettig et al., Proc. Natl. Acad. Sci. USA 85: 3110-3114  
20           (1988); Garin-Chesa et al., Proc. Natl. Acad. Sci. USA 87: 7235-7329 (1990); Rettig et al., Canc. Res. 53: 3327-3335 (1993); Stein et al., in Knapp et al., eds. Leukocyte typing IV-white cell differentiation antigens, pp 412-415 (Oxford University Press, N.Y. 1989), pp. 412-415; Möbius et al., J.  
25           Exp. Immunol. 74: 431-437 (1988)). In view of the potential significance of FAP $\alpha$ /CD26 coassociation, tissue distribution was reexamined, via side by side immunohistochemical staining of normal tissues and lesional tissues known to contain FAP $\alpha$ <sup>+</sup> fibroblasts or FAP $\alpha$ <sup>+</sup> malignant cells.

30           To test the samples, they were embedded in OCT compound, frozen in isopentane precooled in liquid nitrogen, and stored at -70°C until used. Five micrometer thick sections were cut, mounted on poly-L-lysine coated slides, air dried, and fixed in cold acetone (4°C, for 10 minutes). The sections were then tested with mAbs (10-20 ug/ml), using the well known avidin-biotin immuno-peroxidase method, as described by, e.g., Garin-Chesa et al., J. Histochem. Cytochem. 37: 1767-

1776 (1989); Garin-Chesa et al., Proc. Natl. Acad. Sci. USA  
87: 7235-7239 (1990); Rettig et al., Canc. Res. 53: 3327-3335  
(1993); Garin-Chesa et al., Am. J. Pathol. 142: 557-567.

5           The results are shown in figure 2. Breast, colorectal,  
pancreas and lung carcinomas showed strong expression of FAP $\alpha$   
and no CD26 was found (see figures 2A and 2B). Five FAP $\alpha^+$   
sarcomas, including malignant fibrous histiocytoma (figures 2C  
and 2D), were tested, and there was no expression of CD26.  
Examination of reactive fibroblasts of healing dermal wounds  
10          (figures 2E, 2F), showed abundant expression of both FAP $\alpha$  and  
CD26. The three renal carcinomas tested (figures 2G, 2H),  
showed expression of CD26 in malignant epithelium. FAP $\alpha$  was  
absent from malignant epithelial cells, and showed low  
expression in the stroma of these carcinomas.

15          Example 9

A mammalian cell line, transfected with a FAP $\alpha$  encoding  
cDNA, was prepared.

Human embryonic kidney cell line 293 is well known and  
widely available from, e.g., the American Type Culture  
20          Collection.

Samples of 293 were maintained, in an incubator, at 37°C,  
in an atmosphere of 95% air, and 5% CO<sub>2</sub>. The cells were  
cultured in a 50:50 mixture of Dulbecco's modified minimal  
essential medium and Ham's F12 medium, augmented with 10%  
25          fetal bovine serum, penicillin and streptomycin. Following  
the procedures described by Ustar et al., Eur. Mol. Biol. J.  
1991, and/or Park et al., J. Biol. Chem. 169: 25646-25654  
(1994), both of which are incorporated by reference, cDNA for  
FAP $\alpha$  (i.e., SEQ ID NO: 1), was transfected into the 293 cells.  
30          Details of the cDNA vector are provided, *supra* (pFAP.38).  
Transfectants were selected for resistance to antibiotics (200  
ug/ml Geneticin), and were then maintained in selection  
medium, containing Geneticin.

Individual colonies of resistant cells were picked, grown  
35          to confluence in 6 well tissue culture plates, and were tested  
for FAP $\alpha$  expression in an immunofluorescence assay (IFA),  
using FAP $\alpha$  specific monoclonal antibody F19 as described

supra.

Those colonies which expressed FAP $\alpha$  were expanded, and monitored by indirect IFA and cytofluorometric analysis, also as set forth, supra.

5 The IFAs were positive for the transfectants, referred to hereafter as cell line 293-FAP, but were negative for parental line 293.

Example 10

10 In order to confirm that recombinant FAP $\alpha$  was, in fact, being produced, a series of immunoprecipitation experiments were carried out. These followed the methods of Park, et al., supra, and Rettig et al., Canc. Res. 53: 3327-3335 (1993), both of which are incorporated by reference. Essentially,  $^{35}$ [S] methionine labelled cell extracts were combined with 15 monoclonal antibody F19, in the manner described supra. Precipitates were then boiled in extraction buffer and run on SDS-PAGE gels, using, as a negative control, mouse IgG1. Both cell line 293-FAP, and non transfected line 293 were tested. The results indicated clearly, that recombinant FAP $\alpha$  was 20 produced by the transfected cell line 293-FAP. This was determined by immunoprecipitation analyses, using FAP $\alpha$  specific monoclonal antibody F19.

Example 11

25 The ability to produce recombinant FAP $\alpha$  permitted further study of the molecule's properties. Specifically, given the structural features outlined in the prior examples, experiments were designed to determine if FAP $\alpha$  possesses enzymatic activities. The experiments were designed to test whether or not FAP $\alpha$  had extracellular matrix (ECM) protein 30 degrading activity.

Extracts of 293-FAP cells were prepared, using an extraction buffer (0.15M NaCl, 0.05M Tris-HCl, pH 7.4, 10 mM MgCl<sub>2</sub>, 1 percent Triton X-114), were cleared by centrifugation (4,000xg, 10 minutes at 4°C), and phase partitioned at 37°C 35 for 10-20 minutes. This was followed by further centrifugation (4000xg, 20 minutes at 20-25°C). Detergent phases were diluted with buffer (0.15 M NaCl, 0.05 M Tris-HCl

pH 7.4, 5 mM CaCl<sub>2</sub>, 5 mM MgCl<sub>2</sub>, 0.75% Empigen BB), and separated on concanavalin A-Sepharose following Rettig et al., *supra*. Any concanavalin A bound fractions were eluted with 0.25M methyl- $\alpha$ -D-mannopyranoside in elution buffer (0.15 M NaCl, 0.05 M Tris-HCl, pH 7.4, 5mM CaCl<sub>2</sub>, 5 mM MgCl<sub>2</sub>, 0.1% Triton X-100), mixed with zymography sample buffer (0.25 M Tris-HCl, pH 6.8, 8% SDS, 40% glycerol, 0.01% bromophenol blue), at a 3:1 ratio, and used for further analysis.

Aliquots of sample were loaded onto polyacrylamide gels containing 0.1% of either of gelatin or casein. Electrophoresis was then carried out in a Biorad Mini-Protein II system, at 20 mA constant current for 1.5 - 2 hours, until the bromophenol blue dye fronts of samples had reached the lower end of the gel. The gel was removed and incubated for one hour at 20-25°C in a 2.5% aqueous solution of Triton X-100 on a rotary shaker. The Triton X-100 solution was decanted, and replaced with enzyme buffer (0.05M Tris-HCl, pH 7.5, 0.2M NaCl, 5 mM CaCl<sub>2</sub>, 5 mM MgCl<sub>2</sub>, 0.02% Brij 35). The gel was then incubated at 37°C or 41°C, followed by staining or destaining at room temperature. Gels were stained with 0.5% of Coomassie Brilliant Blue G-250 in an aqueous solution of 30% methanol and 10% acetic acid for 15, 30, and 60 minutes, respectively. Subsequently, gels were incubated for 15 minutes in an aqueous solution of 30% CH<sub>3</sub>OH and 5% glycerol, followed by drying between sheets of cellophane.

Gelatinase activity was evaluated in accordance with Kleiner et al., Anal. Biochem. 218: 325-329 (1994), incorporated by reference in its entirety. This is a routine assay used to determine whether or not a protease capable of digesting gelatin is present. Labelled molecular weight standard were run on the same gels, under reducing conditions, for molecular weight determinations.

Proteolytic activity for defined amino acid sequence motifs were tested, using a well known membrane overlay assay. See Smith et al, Histochem. J. 24(9): 637-647 (1992), incorporated by reference. Substrates were Ala-Pro-7-amino-4-trifluoromethyl coumarin, Gly-Pro-7-amino-4-trifluoromethyl

coumarin, and Lys-Pro-7-amino-4-trifluoromethyl coumarin.

The results of these experiments are depicted, in part, in figure 3. This figure shows zymographic detection of gelatin degrading activity, in the cell extracts. See Kleiner et al., supra. A protein species of approximately 170 kilodaltons, as determined by SDS-PAGE, was observed to have gelatin degrading activity. This species, which was found in the 293-FAP cell line, but not in untransfected 293 cells, is thus identified as FAP $\alpha$ . The molecular weight is consistent with a dimer, i.e., a dimeric FAP $\alpha$  molecule.

The proteolytic activity described herein where gelatin is the substrate, was not observed when casein was the substrate.

15       Example 12

Further studies were then undertaken in order to characterize the 170 kD FAP $\alpha$  dimer further. Specifically, the experiments described in example 11 were repeated, except that 5% of 2-mercaptoethanol or 5  $\mu$ M iodoacetamide was added to the extracts prior to SDS-PAGE, or ethylenediamine N,N,N',N'-tetraacetic acid (10 mM) was added to the incubation buffer used for gelatin zymography. None of these treatments abolished the enzymatic activity. In contrast, heating at 100°C for five minutes prior to SDS-polyacrylamide gel electrophoresis abolished the gelatin-degrading activity.

Further work, using a membrane overlay assay, described by, e.g., Smith et al., Histochem J. 24(9): 643-647 (1992), incorporated by reference, revealed that the FAP $\alpha$  dimers were able to cleave all of the Ala-Pro, Gly-Pro, and Lys-Pro dipeptides tested.

In further experiments, a fusion protein was produced which comprised the extracellular domains of both FAP $\alpha$  and murine CD8 proteins. This chimeric protein was produced in a baculovirus system in insect cells. The chimeric protein exhibited the same enzymatic activity as FAP $\alpha$ , using the model discussed *supra*.

30       Example 13

Two quantitative assays for FAP $\alpha$  enzyme activity were developed using Ala-Pro-7-amino-4-trifluoromethyl coumarin (Ala-Pro-AFC) as the substrate. In the first assay format, membrane extracts of FAP $\alpha$ -expressing cells were mixed with a 5-10 fold volume of reaction buffer (100mM NaCl, 100mM Tris pH 7.8), and added to an equal volume of 0.5mM Ala-Pro-AFC in reaction buffer followed by an incubation for one hour at 37°C. Release of free AFC was then measured in a fluorimeter using a 395nm excitation / 530nm emission filter set. The membrane extracts analyzed in this assay format were derived from either 293-FAP $\alpha$  cells (293 cells stably transfected with vector FAP.38 described supral) or HT1080-FAP $\alpha$  cells (HT1080 cells stably transfected with vector FAP.38). Negative control experiments assessing FAP $\alpha$ -specific activities were carried out with membrane extracts prepared from the respective parental 293 or HT1080 cell lines. In the second assay, FAP $\alpha$  was isolated from 293-FAP $\alpha$  or HT1080-FAP $\alpha$  membrane extracts via an antibody specific for FAP $\alpha$ . Ninety-six well ELISA plates were coated overnight at 4°C with 1ug/ml F19 monoclonal antibody in phosphate-buffered saline (PBS). In the case of CD8-FAP $\alpha$  discussed infra plates were coated with F19 antibody as above or with 1ug/ml rat anti-mouse CD8 overnight at 4°C. Wells were then washed with wash buffer (PBS, 0.1% Tween 20). Excess binding sites were blocked with blocking buffer (5% bovine serum albumin in PBS) for 1 hour at room temperature. Blocking buffer was removed; membrane extracts of 293-FAP $\alpha$  expressing cells or control cells were added and incubated for 1 hour at room temperature. The unbound material was removed, wells were washed with wash buffer, and FAP $\alpha$  activity was assayed using 100 ul Ala-Pro-AFC (0.5 mM Ala-Pro-AFC in reaction buffer) for one hour at 37°C. Release of free AFC was measured as above. Binding of mab F19 to FAP $\alpha$  did not measurably affect its enzymatic activity.

Example 14

Using assays for FAP $\alpha$  enzyme activity, described supra an inhibitor of FAP $\alpha$  enzymatic activity has been identified. This inhibitor is (S)-Valylpyrrolidine-2(R)-boronic acid (Snow

et al., J. Am. Chem Soc.(1994) 116:10860-10869), referred to here as ValboroPro. ValboroPro inhibits cleavage of Ala-Pro-AFC by FAP $\alpha$  with an IC<sub>50</sub> of 0.11 uM. ValboroPro also inhibits the gelatinolytic activity of FAP $\alpha$  at a concentration of 100 uM. The specificity of ValboroPro for FAP $\alpha$  was demonstrated in tests with an unrelated serine protease, trypsin. No inhibition of bovine trypsin by ValboroPro (up to 100 uM) was observed when assayed with carbobenzoxy-L-valinyl-glycanyl-L-arginyl-4-nitranilide acetate as substrate.

10       Example 15

The identification of specific, structural requirements for the enzymatic activities of FAP $\alpha$  facilitates the development of molecules which can bind to and/or inhibit FAP $\alpha$ . To examine whether the serine residue at position 624 of the predicted amino acid sequence of FAP $\alpha$  polypeptide is critical for its enzymatic function, site-directed mutagenesis according to Zoller, et al DNA 3:479-488 (1984) was performed using standard polymerase chain reaction methods. The TCC codon coding for serine 624 in the FAP $\alpha$  cDNA was replaced with GCG, resulting in alanine at this position. The altered DNA was reintroduced into the FAP.38 vector and transfected into 293 cells as described *supra*. Geneticin-resistant colonies were selected and examined by indirect IFA for FAP $\alpha$  expression using mAb F19 as well as other FAP $\alpha$  specific antibodies described by Rettig, et al., J. Cancer 58:385-392 (1994) as set forth, *supra*. No differences in binding of the anti-FAP $\alpha$  antibodies to the mutant FAP $\alpha$  expressing cells were observed as compared to wild type FAP $\alpha$  transfected cells. The presence of the mutation was confirmed through amplification of genomic DNA and restriction enzyme digestion performed with several clones of transfected cells. To assess the enzymatic activity of mutant FAP $\alpha$ , the following tests were performed. Membrane extracts were prepared from three independent positive clones and equal amounts of FAP $\alpha$  protein (as determined in a double-determinate ELISA assay using two anti-FAP $\alpha$  antibodies that recognize distinct FAP $\alpha$  epitopes) were examined in the gelatinolytic and Ala-Pro-AFC capture assays.

Both the gelatinolytic activity and the activity in the capture assay of isolated mutant FAP $\alpha$  were reduced to undetectable levels compared to wild type FAP $\alpha$ , confirming the role of the canonical serine in the catalytic triad for both observed enzymatic activities.

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Example 16

A fusion protein was generated to obtain secreted, water-soluble FAP $\alpha$  enzyme. In this fusion protein, the extracellular domain of CD8, consisting of the first 189 amino acids of murine CD8, was linked to the extracellular domain of FAP $\alpha$  (amino acids 27 to 760), as described by Lane et al., J. Exp. Med. 177:1209 (1993) using standard polymerase chain reaction protocols and inserted in commercially available pVL1393 vector. Transfection of Sf9 cells with this vector and amplification of the resulting recombinant baculovirus were performed as described (Baculovirus Expression Vectors, O'Reilly, Miller, and Luckow, Oxford University Press, 1994). The CD8-FAP fusion protein was isolated in a two step purification from the spent medium of High Five™ cells infected with CD8-FAP $\alpha$  baculovirus for four days. Cells and virus were removed by ultracentrifugation, the supernatant was passed through a column containing Heparin-Sepharose (Pharmacia) and eluted stepwise with 0.6, 1.0, and 2.0 M NaCl in 10mM phosphate, pH 7. Active fractions from the 1.0 and 2.0 M eluates were pooled and concentrated using an YM-10 filter and 26/60 Superdex-200 gel filtration column. Activity was observed in a high molecular weight peak which, when subjected to N-terminal gas phase sequencing, was confirmed to be CD8-FAP $\alpha$ . In gelatinolytic assays, activity greater than 200kD in the gelatinolytic assay was detected when purified CD8-FAP $\alpha$  was tested, consistent with the higher predicted molecular weight of the fusion protein.

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Example 17

The presence of structural and functional homologues in non-human species has been ascertained. For example, the cDNA for mouse FAP $\alpha$  has been cloned and characterized. Examination of the predicted amino acid sequence of the homologous mouse

FAP $\alpha$  cDNA sequence (EMBL accession number Y10007) reveals a high degree of conservation of FAP $\alpha$  across species. The two proteins are 89% identical and the catalytic triad is conserved between human FAP $\alpha$  and mouse FAP $\alpha$ . The high degree of conservation and similar tissue expression suggests that FAP $\alpha$  from nonhuman sources may be functionally equivalent to human FAP $\alpha$ . This conclusion is confirmed by the finding that a CD8-murine FAP $\alpha$  fusion protein similar in design to CD8-human FAP $\alpha$  also demonstrates the expected dipeptidylpeptidase enzymatic activity using Ala-Pro-AFC as substrate.

The foregoing examples describe an isolated nucleic acid molecule which codes for fibroblast activating protein alpha ("FAP $\alpha$ "), as well as dimeric forms of the molecule, and uses thereof. The expression product of the sequence in COS-1 is a protein which, on SDS-PAGE of boiled samples, shows a molecular weight of about 88 kd. Deduced amino acid sequence, as provided in SEQ ID NO: 1, for one form of the molecule, yields a molecular weight of about 88 kd.

It should be noted that there is an apparent discrepancy in molecular weight in that the COS-1 isolate is glycosylated, while molecular weight from deduced amino acid sequences does not account for glycosylation. Membrane proteins are known to exhibit aberrant migration in gel systems, however, which may explain the difference observed here.

Also a part of the invention are chimeric and fusion proteins, which comprise a portion of FAP $\alpha$  which contain the molecule's catalytic domain, and additional, non FAP $\alpha$  components. The FAP $\alpha$  catalytic domain *per se* is also a part of the invention.

It is to be understood that, as described, FAP $\alpha$  may be glycosylated, with the type and amount of glycosylation varying, depending upon the type of cell expressing the molecule. The experiment described herein shows this. This is also true for the dimeric form of the molecule, first described herein, having a molecular weight of about 170 kilodaltons as determined by SDS-PAGE of unboiled samples.

The invention also comprehends the production of

expression vectors useful in producing the FAP $\alpha$  molecule. In their broadest aspect, these vectors comprise the entire FAP $\alpha$  coding sequence or portions thereof, operably linked to a promoter. Additional elements may be a part of the expression vector, such as protein domains fused to the FAP $\alpha$  protein or protein portions ("fusion protein") genes which confer antibiotic resistance, amplifiable genes, and so forth.

The coding sequences and vectors may also be used to prepare cell lines, wherein the coding sequence or expression vector is used to transfect or to transform a recipient host. The type of cell used may be prokaryotic, such as *E. coli*, or eukaryotes, such as yeast, CHO, COS, or other cell types.

The identification of nucleic acid molecules such as that set forth in SEQ ID NO: 1 also enables the artisan to identify and to isolate those nucleic acid molecules which hybridize to it under stringent conditions. "Stringent condition" as used herein, refers to those parameters set forth supra, whereby both murine and hamster sequences were also identified. It will be recognized by the skilled artisan that these conditions afford a degree of stringency which can be achieved using parameters which vary from those recited. Such variance is apprehended by the expression "stringent conditions".

The ability of nucleic acid molecules to hybridize to complementary molecules also enables the artisan to identify cells which express FAP $\alpha$ , via the use of a nucleic acid hybridization assay. One may use the sequences described in the invention to hybridize to complementary sequences, and thus identify them. In this way, one can target mRNA, e.g., which is present in any cell expressing the FAP $\alpha$  molecule.

It is of course understood that the nucleic acid molecules of the invention are also useful in the production of recombinant FAP $\alpha$ , in both monomeric and dimeric form. The examples clearly show that host cells are capable of assembling the dimeric forms. The recombinant protein may be used, e.g., as a source of an immunogen for generation of antibodies akin to known mAb F19, and with the same uses. Similarly, the recombinant protein, and/or cells which express

the molecule on their surface, may be used in assays to determine antagonists, agonists, or other molecules which interact with molecules having FAP $\alpha$  activity. Such substances may be, but are not necessarily limited to, substrates, inhibiting molecules, antibodies, and so forth. The molecules having FAP $\alpha$  activity may be, e.g., the monomeric or dimeric forms of FAP $\alpha$ , derivatives containing the catalytic domain, and so forth. The molecule having FAP $\alpha$  activity may be pure, or in the form of a cell extract, such as a transformed or transfected cell, which has received an FAP $\alpha$  active gene. Both prokaryotes and eukaryotes may be used. This last feature of the invention should be considered in light of the observed structural resemblances to membrane bound enzymes. This type of molecule is associated with certain properties which need not be described in detail here. It will suffice to say that inhibition or potentiation of these properties as associated with FAP $\alpha$  is a feature of this invention. For example, one may identify substrates or the substrate for FAP $\alpha$  molecules, via the use of recombinant cells or recombinant FAP $\alpha$  per se. The substrates can be modified to improve their effect, to lessen their effect, or simply to label them with detectable signals so that they can be used, e.g., to identify cells which express FAP $\alpha$ . Study of the interaction of substrate and FAP $\alpha$ , as well as that between FAP $\alpha$  and any molecule whatsoever, can be used to develop and/or to identify agonists and antagonists of the FAP $\alpha$  molecule.

Also a feature of the invention are isolated, dimeric FAP $\alpha$  molecules which have a molecular weight of about 170 kilodaltons as determined by SDS-PAGE, their use as an enzymatic cleaving agent, and other uses as are described herein. Enzymatically active forms of FAP $\alpha$  may also be produced as recombinant fusion proteins, such as soluble fusion proteins comprising the catalytic domain of FAP $\alpha$  and other protein domains with suitable biochemical properties, including secretory signals, protease cleavage sites, tags for purification, and other elements known to the artisan.

Exemplary are CD8 peptide sequences, such as are described supra. The fact that FAP $\alpha$  has particular properties, as described herein, permits the identification of the molecule on cells expressing them. In turn, because the FAP $\alpha$  molecule is associated with tumors and tumor stromal cells, targeting of FAP $\alpha$  with therapeutic agents serves as a way to treat cancerous or precancerous condition, by administering sufficient therapeutic agent to alleviate cancer load.

The experiments showing the proteolytic properties of FAP $\alpha$  lead to yet a further aspect of the invention. It is well known that proteases which degrade extracellular matrix, or "ECM" proteins have an important role on certain aspects of tumor growth, including their effect on tumor cell invasion, tumor blood vessel formation (i.e., neoangiogenesis), and tumor metastasis. Collagens are of special interest vis-a-vis the substrates of proteases, as the collagens are an important part of the ECM. The fact that FAP $\alpha$  digests ECM suggests a therapeutic role for inhibitors of the molecule. "Inhibitors", as used herein, refers to molecules which interfere with FAP $\alpha$  enzyme function. Specifically excluded from such inhibitors is the monoclonal antibody F19. This mAb is known to bind to but not inhibit the enzyme function of FAP $\alpha$ , and hence it is not an inhibitor. The art is quite well versed with respect to monoclonal antibodies which both bind to and inhibit enzymes. Further examples of such inhibitors would include, e.g., substrate derivatives, such as modified collagen molecules, which interfere with the active site or sites of the FAP $\alpha$  molecule. Other suitable inhibitors will be apparent to the skilled artisan, and need not be listed here. In addition, the recombinant FAP $\alpha$  proteins and FAP $\alpha$ -transfected cell lines described supra can be employed in an enzymatic screening assay, using the substrate described supra or other suitable substrates, to identify inhibitors from any compound library. The identification of substances which interact with FAP $\alpha$  active molecules thereby leads to therapeutic treatment of conditions where a subject exhibit abnormal FAP $\alpha$  activity. Specifically, an amount of an

appropriate substance, be it an inhibitor (e.g., a collagen derivative, S-Valyl-pyrrolidine-2(R)-boronic acid), an agonist or an antagonist is administered to a subject in an amount sufficient to normalize FAP $\alpha$  activity.

5 Other aspects of the invention will be clear to the skilled artisan, and need not be set forth here.

The terms and expressions which have been employed are used as terms of description and not of limitation, and there is no intention in the use of such terms and expressions of excluding any equivalents of the features shown and described or portions thereof, it being recognized that various modifications are possible within the scope of the invention.

## (1) GENERAL INFORMATION:

(i) APPLICANTS: Zimmermann, Rainer; Park, John E.;  
Rettig, Wolfgang; Old, Lloyd J.  
(ii) TITLE OF INVENTION: ISOLATED DIMERIC FIBROBLAST ACTIVATION  
PROTEIN ALPHA, AND USES THEREOF

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(iii) NUMBER OF SEQUENCES: 2

10

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15

(v) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Diskette, 3.5 inch, 2.0 MB storage  
(B) COMPUTER: IBM PS/2  
(C) OPERATING SYSTEM: PC-DOS  
(D) SOFTWARE: Wordperfect

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(C) CLASSIFICATION: 435

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(B) FILING DATE: 20-APRIL-1994

35

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## (2) INFORMATION FOR SEQ ID NO: 1:

## (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 2815 Base pairs  
 (B) TYPE: nucleic acid  
 5 (C) STRANDEDNESS: double  
 (D) TOPOLOGY: linear

## (xi) SEQUENCE DESCRIPTION: SEQ. ID NO: 1:

10	AAGAACGCC CAAAAATCTG TTTCTAATTT TACAGAAATC TTTGAAACT TGGCACGGTA	60
	TTCAAAAGTC CGTGGAAAGA AAAAAACCTT GTCCCTGGCTT CAGCTTCAA CTACAAAGAC	120
	AGACTTGGTC CTTTCAACG GTTTACAG ATCCAGTGAC CCACGCTCTG AAGACAGAAAT	180
	TAGCTAACTT TCACAAACAT CTGGAAAAAT GAAGACTTGG GAAAAATCG TATTGGAGT	240
	TGCCACCTCT GCTGTGCTTG CCTTATTGGT GATGTGCATT GTCTTACGCC CTTCAAGAGT	300
15	TCATAACTCT TAAGAAAAATA CAATGAGAGC ACTCACACTG AAGGATATT TAAATGGAAC	360
	ATTTCTTAT AAAACATTTC TTCCAAACTG GATTTCAGGA CAAGAATATC TTCAATCAATC	420
	TGCAGATAAC AAATATAGTAC TTATATAATAT TGAAACAGGA CAATCATATA CCATTTGAG	480
	TAATAGAACC ATGAAAAGTG TGAATGCTTC AAATTACGGC TTATCACCTG ATGGCAATT	540
	TGTATATCTA GAAAGTGATT ATTCAAGCT TTGGAGATAC TCTTACACAG CAACATATTA	600
20	CATCTATGAC CTTAGCAATG GAGAATTGT AAGAGGAAAT GAGCTTCCTC GTCCAATTCA	660
	GTATTTATGC TGGTCGCCTG TTGGGAGTAA ATTAGCATAT GTCTATCAA ACAATATCTA	720
	TTTGAACAA AGACCAGGAG ATCCACCTT TCAATAACA TTTAATGGAA GAGAAAATAA	780
	AAATTTTAAT GGAATCCCAG ACTGGTTTA TGAAGAGGAA ATGCTTCCTA CAAAATATGC	840
	TCTCTGGTGG TCTCTTAATG GAAAATTTT GGCATATGCG GAATTTAATG ATAAGGATAT	900
25	ACCAAGTTTGCCT ATTATGGCGA TGAACAATAT CCTAGAACAA TAAATATTCC	960
	ATACCCAAAG GCTGGAGCTA AGAATCCCGT TGTTCGGATA TTATTTATCG ATACCACTTA	1020
	CCCTGCGTAT GTAGGTCCCC AGGAAGTGCC TGTTCCAGCA ATGATAGCCT CAAGTGATTA	1080
	TTATTTCACTG TGGCTCACGT GGGTTACTGA TGAACGAGTA TGTTGAGT GGCTAAAAG	1140
	AGTCCAGAAT GTTCCGGTCC TGTCTATATG TGACTTCAGG GAAGACTGGC AGACATGGGA	1200
30	TTGTCCAAAG ACCCAGGGAGC ATATAGAAGA AAGCAGAACT GGATGGGCTG GTGGATTCTT	1260
	TGTTTCAAGA CCAGTTTCA GCTATGATGC CATTCTGTAC TACAAAATAT TTAGTGACAA	1320
	GGATGGCTAC AAACATATTC ACTATATCAA AGACACTGTG GAAAATGCTA TTCAAATTAC	1380
	AAGTGGCAAG TGGGAGGCCA TAAATATATT CAGAGTAACA CAGGATTAC TGTTTATTC	1440
	TAGCAATGAA TTGAAGAAT ACCCTGGAAG AAGAAACATC TACAGAATTA GCATTGGAG	1500
35	CTATCCCA AGCAAGAAGT GTGTTACTTG CCATCTAAGG AAAGAAAGGT GCCAATATTA	1560
	CACAGCAAGT TTCAGGGACT ACGCCAAGTA CTATGCACTT GTCTGCTACG GCCCAGGCAT	1620
	CCCCATTTCAC ACCCTTCATG ATGGACGCAC TGATCAAGAA ATTAAAATCC TGGAGAAAA	1680

25

CAAGGAATTG GAAAATGCTT TGAAAAATAT CCAGCTGCCT AAAGAGGAAA TTAAGAAACT 1740  
 TGAAGTAGAT GAAATTACTT TATGGTACAA GATGATTCTT CCTCCTCAAT TTGACAGATC 1800  
 AAAGAAGTAT CCCTTGCTAA TTCAGTGTG TGCGAGTCAGA GTGTAAGGTC 1860  
 5 TGTATTTGCT GTTAATTGGA TATCTTATCT TGCAAGTAAG GAAGGGATGG TCATTGCCTT 1920  
 GGTGGATGGT CGAGGAACAG CTTTCCAAGG TGACAAACTC CTCTATGCAG TGTATCGAA 1980  
 GCTGGGTGTT TATGAAGTTG AAGACCAGAT TACAGCTGTC AGAAAATTCA TAGAAATGGG 2040  
 TTTCATTGAT GAAAAAAGAA TAGCCATATG GGGCTGGTCC TATGGAGGAT ACGTTTCATC 2100  
 ACTGGCCCTT GCATCTGGAA CTGGCTTTT CAAATGTGGT ATAGCAGTGG CTCCAGTCTC 2160  
 CAGCTGGGAA TATTACCGCT CTGTCTACAC AGAGAGATTG ATGGGTCTCC CAACAAAGGA 2220  
 10 TGATAATCTT GAGCACTATA AGAAATCAAC TGTGATGGCA AGACCAGAAAT ATTTCAGAAA 2280  
 TGTAGACTAT CTTCTCATCC ACAGAACAGC AGATGATAAT GTGCACTTTC AAAACTCAGC 2340  
 ACAGATTGCT AAAGCTCTGG TTAATGCACA AGTGGATTTC CAGGCAATGTG GGTACTCTGA 2400  
 CCAGAACAC GGCTTATCCG CCCTGTCCAC GAACCACTTA TACACCCACA TGACCCACTT 2460  
 CCTAAAGCAG TGTTTCTCTT TGTCAGACTA AAAACGATGC AGATGCAAGC CTGTATCAGA 2520  
 15 ATCTGAAAC CTTATATAAA CCCCTCAGAC AGTTTGCTTA TTTTATTTT TATGTTGTAA 2580  
 AATGCTAGTA TAAACAAACA ATTAAATGTT GTTCTAAAGG CTGTTAAAAA AAAGATGAGG 2640  
 ACTCAGAACT TCAAGCTAA TATTGTTAC ATTTCTGGT ACTCTGTGAA AGAAGAGAAA 2700  
 AGGGAGTCAT GCATTTGCT TTGGACACAG TGTTTATCA CCTGTTCACT TGAAGAAAAA 2760  
 TAATAAGTC AGAAGTCAA AAAAAAAAAA AAAAAAAAAA AAAGGGCCCG CTCGA 2815  
 20

## (2) INFORMATION FOR SEQ ID NO: 2:

## (i) SEQUENCE CHARACTERISTICS:

25 (A) LENGTH: 760 amino acids  
 (B) TYPE: amino acid  
 (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

30 Met Lys Thr Trp Val Lys Ile Val Phe Gly Val Ala Thr Ser Ala Val  
                       5                     10                    15  
 Leu Ala Leu Leu Val Met Cys Ile Val Leu Arg Pro Ser Arg Val His  
                       20                    25                    30  
 35 Asn Ser Glu Glu Asn Thr Met Arg Ala Leu Thr Leu Lys Asp Ile Leu  
                       35                    40                    45  
 Asn Gly Thr Phe Ser Tyr Lys Thr Phe Phe Pro Asn Trp Ile Ser Gly

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	50	55	60
	Gln Glu Tyr Leu His Gln Ser Ala Asp Asn Asn Ile Val Leu Tyr Asn		
	65	70	75
	Ile Glu Thr Gly Gln Ser Tyr Thr Ile Leu Ser Asn Arg Thr Met Lys		80
5	85	90	95
	Ser Val Asn Ala Ser Asn Tyr Gly Leu Ser Pro Asp Arg Gln Phe Val		
	100	105	110
	Tyr Leu Glu Ser Asp Tyr Ser Lys Leu Trp Arg Tyr Ser Tyr Thr Ala		
	115	120	125
10	Thr Tyr Tyr Ile Tyr Asp Leu Ser Asn Gly Glu Phe Val Arg Gly Asn		
	130	135	140
	Glu Leu Pro Arg Pro Ile Gln Tyr Leu Cys Trp Ser Pro Val Gly Ser		
	145	150	155
	Lys Leu Ala Tyr Val Tyr Gln Asn Asn Ile Tyr Leu Lys Gln Arg Pro		160
15	165	170	175
	Gly Asp Pro Pro Phe Gln Ile Thr Phe Asn Gly Arg Glu Asn Lys Ile		
	180	185	190
	Phe Asn Gly Ile Pro Asp Trp Val Tyr Glu Glu Glu Met Leu Pro Thr		
	195	200	205
20	Lys Tyr Ala Leu Trp Trp Ser Pro Asn Gly Lys Phe Leu Ala Tyr Ala		
	210	215	220
	Glu Phe Asn Asp Lys Asp Ile Pro Val Ile Ala Tyr Ser Tyr Tyr Gly		
	225	230	235
	Asp Glu Gln Tyr Pro Arg Thr Ile Asn Ile Pro Tyr Pro Lys Ala Gly		240
25	245	250	255
	Ala Lys Asn Pro Val Val Arg Ile Phe Ile Ile Asp Thr Thr Tyr Pro		
	260	265	270
	Ala Tyr Val Gly Pro Gln Glu Val Pro Val Pro Ala Met Ile Ala Ser		
	275	280	285
30	Ser Asp Tyr Tyr Phe Ser Trp Leu Thr Trp Val Thr Asp Glu Arg Val		
	290	295	300
	Cys Leu Gln Trp Leu Lys Arg Val Gln Asn Val Ser Val Leu Ser Ile		
	305	310	315
	Cys Asp Phe Arg Glu Asp Trp Gln Thr Trp Asp Cys Pro Lys Thr Gln		320
35	325	330	335
	Glu His Ile Glu Glu Ser Arg Thr Gly Trp Ala Gly Gly Phe Phe Val		
	340	345	350

Ser Arg Pro Val Phe Ser Tyr Asp Ala Ile Ser Tyr Tyr Lys Ile Phe  
                  355                 360                 365  
 Ser Asp Lys Asp Gly Tyr Lys His Ile His Tyr Ile Lys Asp Thr Val  
                  370                 375                 380  
 5 Glu Asn Ala Ile Gln Ile Thr Ser Gly Lys Trp Glu Ala Ile Asn Ile  
                  385                 390                 395                 400  
 Phe Arg Val Thr Gln Asp Ser Leu Phe Tyr Ser Ser Asn Glu Phe Glu  
                  405                 410                 415  
 Glu Tyr Pro Gly Arg Arg Asn Ile Tyr Arg Ile Ser Ile Gly Ser Tyr  
 10               420                 425                 430  
 Pro Pro Ser Lys Lys Cys Val Thr Cys His Leu Arg Lys Glu Arg Cys  
                  435                 440                 445  
 Gln Tyr Tyr Thr Ala Ser Phe Ser Asp Tyr Ala Lys Tyr Tyr Ala Leu  
                  450                 455                 460  
 15 Val Cys Tyr Gly Pro Gly Ile Pro Ile Ser Thr Leu His Asp Gly Arg  
                  465                 470                 475                 480  
 Thr Asp Gln Glu Ile Lys Ile Leu Glu Glu Asn Lys Glu Leu Glu Asn  
                  485                 490                 495  
 Ala Leu Lys Asn Ile Gln Leu Pro Lys Glu Glu Ile Lys Lys Leu Glu  
 20               500                 505                 510  
 Val Asp Glu Ile Thr Leu Trp Tyr Lys Met Ile Leu Pro Pro Gln Phe  
                  515                 520                 525  
 Asp Arg Ser Lys Lys Tyr Pro Leu Leu Ile Gln Val Tyr Gly Gly Pro  
                  530                 535                 540  
 25 Cys Ser Gln Ser Val Arg Ser Val Phe Ala Val Asn Trp Ile Ser Tyr  
                  545                 550                 555                 560  
 Leu Ala Ser Lys Glu Gly Met Val Ile Ala Leu Val Asp Gly Arg Gly  
                  565                 570                 575  
 Thr Ala Phe Gln Gly Asp Lys Leu Leu Tyr Ala Val Tyr Arg Lys Leu  
 30               580                 585                 590  
 Gly Val Tyr Glu Val Glu Asp Gln Ile Thr Ala Val Arg Lys Phe Ile  
                  595                 600                 605  
 Glu Met Gly Phe Ile Asp Glu Lys Arg Ile Ala Ile Trp Gly Trp Ser  
                  610                 615                 620  
 35 Tyr Gly Gly Tyr Val Ser Ser Leu Ala Leu Ala Ser Gly Thr Gly Leu  
                  625                 630                 635                 640  
 Phe Lys Cys Gly Ile Ala Val Ala Pro Val Ser Ser Trp Glu Tyr Tyr

28

	645	650	655
	Ala Ser Val Tyr Thr Glu Arg Phe Met Gly Leu Pro Thr Lys Asp Asp		
	660	665	670
	Asn Leu Glu His Tyr Lys Asn Ser Thr Val Met Ala Arg Ala Glu Tyr		
5	675	680	685
	Phe Arg Asn Val Asp Tyr Leu Leu Ile His Gly Thr Ala Asp Asp Asn		
	690	695	700
	Val His Phe Gln Asn Ser Ala Gln Ile Ala Lys Ala Leu Val Asn Ala		
	705	710	715
10	Gln Val Asp Phe Gln Ala Met Trp Tyr Ser Asp Gln Asn His Gly Leu		
	725	730	735
	Ser Gly Leu Ser Thr Asn His Leu Tyr Thr His Met Thr His Phe Leu		
	740	745	750
	Lys Gln Cys Phe Ser Leu Ser Asp		
	755	760	

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Where the terms "comprise", "comprises", "comprised" or  
"comprising" are used in this specification, they are to be interpreted as specifying  
the presence of the stated features, integers, steps or components referred to, but  
not to preclude the presence or addition of one or more other feature, integer,  
step, component or group thereof.



We claim:

1. Isolated, dimeric FAP $\alpha$  molecule, having a molecular weight of about 170 kilodaltons as determined by SDS-PAGE, wherein said dimeric FAP $\alpha$  molecule is capable of degrading extracellular matrix proteins.
2. The dimeric FAP $\alpha$  molecule of claim 1, wherein each monomer of said dimeric FAP $\alpha$  molecule consists of the amino acid sequence of SEQ ID NO: 2.
3. The dimeric FAP $\alpha$  molecule of claim 1, produced recombinantly.
4. The dimeric FAP $\alpha$  molecule of claim 3, produced by a eukaryotic cell.
5. Isolated protein consisting of:
  - (i) the FAP $\alpha$  catalytic domain, and
  - (ii) at least one portion of a non FAP $\alpha$  protein.
6. Method for cleaving a terminal dipeptide of formula Xaa-Pro from a molecule, comprising contacting said molecule with a second molecule, said second molecule having FAP $\alpha$  enzymatic activity.

7. The method of claim 6, wherein said second molecule is isolated, dimeric FAP $\alpha$ .
8. The method of claim 6, wherein said second molecule comprises an FAP $\alpha$  catalytic domain.
9. Method for identifying a substance which interacts with a molecule having FAP $\alpha$  activity, comprising combining said molecule with a sample to be tested, and determining any interaction with said molecule as an indication of a molecule which interacts with a molecule having FAP $\alpha$  activity.
10. The method of claim 9, wherein said FAP $\alpha$  molecule is dimeric.
11. The method of claim 9, wherein said molecule comprises a FAP $\alpha$  catalytic domain.
12. The method of claim 9, wherein said substance is an antagonist of FAP $\alpha$  activity.
13. The method of claim 9, wherein said substance is an agonist of FAP $\alpha$  activity.
14. The method of claim 9, wherein said substance is an inhibitor of FAP $\alpha$  activity.
15. The method of claim 9, comprising combining said substance with a cell extract which has FAP $\alpha$  activity.
16. The method of claim 15, wherein said cell extract is an extract of a cell which has been transformed or transfected with a nucleic acid molecule which encodes a molecule with FAP $\alpha$  activity.
17. The method of claim 16, wherein said cell is a

prokaryote.

18. The method of claim 16, wherein said cell is a eukaryote.
19. A method for treating a subject with a pathological condition characterized by an increased level of FAP $\alpha$  activity relative to a normal level, said increase in FAP $\alpha$  activity being characterized by an increase in extracellular matrix protein degrading activity, comprising administering to a subject in need thereof an amount of a substance which interacts with FAP $\alpha$  molecules or molecules having extracellular matrix protein degrading activity, sufficient to reduce the FAP $\alpha$  activity level in said subject.
20. The method of claim 19, comprising administering an inhibitor of FAP $\alpha$  activity.
21. The method of claim 20, wherein said inhibitor is a collagen derivative.
22. The method of claim 20, wherein said inhibitor is (S)-valyl-pyrrolidine-2(R) -boronic acid.
23. The method of claim 19, wherein said substance is an agonist of FAP $\alpha$  activity.
24. The method of claim 19, wherein said substance is an antagonist of FAP $\alpha$  activity.
25. A method for determining the ability of a substance to decrease FAP $\alpha$  activity comprising combining a molecule having FAP $\alpha$  activity, said molecule having FAP $\alpha$  activity being characterized as having extracellular matrix protein degrading activity, with Ala-Pro-AFC in the presence and absence of said substance and



determining a level of FAP $\alpha$  activity, wherein a decrease in FAP $\alpha$  activity of said molecule in the presence of said substance indicates that said substance decreases FAP $\alpha$  activity of said molecule.

26. Fusion protein comprising a portion of an FAP $\alpha$  molecule sufficient to retain FAP $\alpha$  activity and a non FAP $\alpha$  amino acid sequence, wherein said fusion protein is water soluble.
  
27. The fusion protein of claim 26, wherein said non FAP $\alpha$  amino acid sequence is an amino acid sequence found in a CD8 protein.
  
28. The fusion protein of claim 27, wherein said CD8 protein is a murine protein.
  
29. The fusion protein of claim 27, wherein said CD8 protein is a human protein.
  
30. The fusion protein of claim 27 comprising amino acid 1 to 189 of murine CD8 linked to amino acids 27 through 760 of FAP $\alpha$ .
  
31. Isolated, dimeric FAP $\alpha$  molecule, having a molecular weight of about 170 kilodaltons as determined by SDS-PAGE, wherein said dimeric FAP $\alpha$  molecule is capable of degrading extracellular matrix proteins and wherein said dimeric FAP $\alpha$  molecule is substantially as herein described with reference to at least one of the accompanying Examples.
  
32. Isolated protein consisting of:
  - (i) the FAP $\alpha$  catalytic domain, and
  - (ii) at least one portion of a non FAP $\alpha$  proteinwherein said isolated protein is substantially as herein described with reference to at least one of the accompanying Examples.



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33. Method for cleaving a terminal dipeptide of formula Xaa-Pro from a molecule, comprising contacting said molecule with a second molecule, said second molecule having FAP $\alpha$  enzymatic activity, wherein said method is substantially as herein described with reference to at least one of the accompanying Examples.
34. Method for identifying a substance which interacts with a molecule having FAP $\alpha$  activity, comprising combining said molecule with a sample to be tested, and determining any interaction with said molecule as an indication of a molecule which interacts with a molecule having FAP $\alpha$  activity wherein said method is substantially as herein described with reference to at least one of the accompanying Examples.
35. A method for determining the ability of a substance to decrease FAP $\alpha$  activity comprising combining a molecule having FAP $\alpha$  activity, said molecule having FAP $\alpha$  activity being characterized as having extracellular matrix protein degrading activity, with Ala-Pro-AFC in the presence and absence of said substance and determining a level of FAP $\alpha$  activity, wherein a decrease in FAP $\alpha$  activity of said molecule in the presence of said substance indicates that said substance decreases FAP $\alpha$  activity of said molecule wherein said method is substantially as herein described with reference to at least one of the accompanying Examples.
36. Fusion protein comprising a portion of an FAP $\alpha$  molecule sufficient to retain FAP $\alpha$  activity and a non FAP $\alpha$  amino acid sequence, wherein said fusion protein is water soluble and wherein said fusion protein is substantially as herein described with reference to at least one of the accompanying Examples.

Dated this 5<sup>th</sup> day of September 2000  
Ludwig Institute for Cancer Research and Boehringer Ingelheim International GmbH

By their Patent Attorneys



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## FIG. 1

FAP	1	MKTWVKIVFGV*ATSAVLALLVMCIVLRPSRVHNSEENTMRALTLKDILN	49
CD26	1	---PW-VLL-LLGAA-LVTIITVPV--LNKGTDATADSRKTY--T-Y-K	50
FAP	50	<u>GTFSYKTFFPNWISGQEYLHQ</u> SADNNIVLYNIETGQSYTILSNRTMKS*	98
CD26	51	N-YRL-LYSLR---DH---YKQ*E---LVF-A-Y-N-SVF-E-S-FDEFG	99
FAP	99	*NASNYGLSPDRQFVYLESDYSKLWRYSYTATYYIYDLSNCEFVRGNELP	147
CD26	100	HSIND-SI---G--IL--YN-V-Q--H---S-D---NKRQLITEERI-	149
		fap-1	
FAP	148	RPIQYLCWSPVGSKLAYVQNNIYLKQRPGDPFQITFNGRENKIFNGIP	197
CD26	150	NNT-WVT----H----WN-D--V-IE-NL-SYR---WT-K-DI-Y---T	199
		fap-2	
FAP	198	<u>DWVYEEEMLPKYALWWSPNGKFLAYAEFNDKDIPVIAYSYYGDE**QYP</u>	245
CD26	200	-----VFSAYS-----T-----Q---TEV-L-E---F-S--SL---	249
FAP	246	RTINIPYPKAGAKNPVVRFIDIIDT***TYPAYVGPQEVPVPAMIASSDYY	292
CD26	250	K-VRV-----V--T-KF-VVN-DSLSSVTNATSIQITA--SMLIG-H-	299
FAP	293	PSWLWTWVTDERVCLQWLKRVONVSVLSICDFREDWQTWDCPKTQEHIIES	342
CD26	300	LCDV--A-Q--IS---R-I--Y--MD---YD-SSGR-N-LVARQ---M-	349
FAP	343	RTGWAGGFFVSRPVFSYDAISYYKIFSDKDGYKHIHYIKDTVENAIQITS	392
CD26	350	T---V-R-RP-E-H-TL-GN-F---I-NEE--R--C-FQIDKKDCTF--K	399
FAP	393	GKWEAINIFRVTQDSLFYSSNEPEEYPGRRNIYRISIGSYPPSKKCVTCH	442
CD26	400	-T--V-G-EAL-S-Y-Y-I---YKGM--G--L-K-QLSD-T*KVT-LS-E	448
FAP	443	LRKERCQYYTASFSDYAKYYALVCYCPGIPISTLHDGRTDQEIKILEENK	492
CD26	449	-NP-----SV---KE---Q-R-S---L-LY---SSVN-KGLRV--D-S	498
		fap-3	
FAP	493	ELENALKNIQLPKEEIKKLEVDEITLWYKMLPPQFDRSKYPLLIQVYG	542
CD26	499	A-DKM-Q-V-M-SKLDLFIILN-TKF--Q-----H--K-----LD--A	548
FAP	543	GPCSQSVRSVPFAVNWISYLASKEGMVIALVDGRGTAFQGDKLLYAVYRKL	592
CD26	549	-----KADT--RL--AT---T-NIIV-SF---SGY---IMH-IN-R-	598
FAP	593	GVYEVEDQITAVRKFIEMGFIDEKRIAIWGSYEIRFITGPCIWNWSFQM	642
CD26	599	-TF-----E-A-Q-SK---V-N-----GGYVTSMVLGSGSVGFK	648
FAP	643	WYSSGSSLQLGILRVCLHRE*IHGSPNKDDNL <del>EHYKNSTV</del> MARA <del>EYFRNV</del>	691
CD26	649	CGIAVAPVSRWEYDSVYT-RYM-L-TPE---D--R-----S---N-KQ-	698
FAP	692	DYLLIHTADDNVHFQNSAQIAKALVNAQVDFQAMWYSDQNHGLSGLSTN	741
CD26	699	E-----Q---S---DVG-----T-ED--IASSTAH	748
FAP	742	*HLYTHMTHFLKQCFSLSD	
CD26	749	Q-I----S--I-----P	

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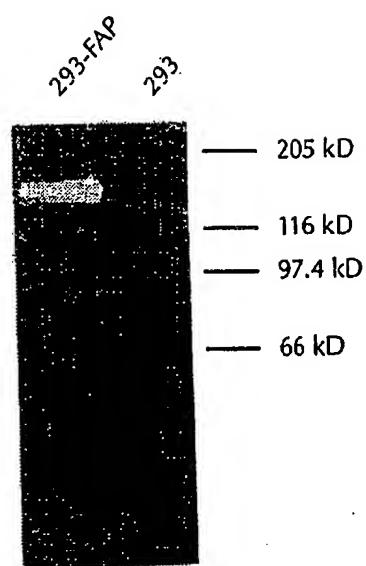
**FIG. 2**

FAP $\alpha$	Breast Cancer A	MFH C	Healing Wound E	Renal Cancer G
CD26	(-) B	(-) D	(+) F	(+) H

Immunohistochemistry (See Kodachromes)

SUBSTITUTE SHEET (RULE 26)

**FIG. 3**



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**Intellectual Property Office of New Zealand**  
**IP Summary Report**

Page: 1 of 1  
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 (iprp02 3.00.02)

<b>(61) Classification:</b>  A61K38/43	Status: 70 Accepted	<b>Version number:</b> 5  <b>IP type:</b> Patent Convention	335543		
	Client Ref: JP801968				
<b>(22) NZ Filing date:</b> 03 May 1989 <b>(30) Priority Data:</b> (31) 96 619280 (32) 18 March 1996 (33) US		<b>(62) Divided out of:</b> 331758 <b>Divisional number(s):</b> 509792 <b>Date actions completed:</b> Application Accepted 15 March 2001 <b>Next renewal date:</b> 03 May 2003			
<b>(71) Applicants:</b> LUDWIG INSTITUTE FOR CANCER RESEARCH, 605 Third Avenue, New York, New York 10158, United States of America <b>BOEHRINGER INGELHEIM INTERNATIONAL GmbH</b> , D-6507 Ingelheim am Rhein, Federal Republic of Germany					
<b>(72) Inventors:</b> Zimmerman, Rainer Park, John E Old, Lloyd J Rettig, Wolfgang <b>Contact:</b> BALDWIN SHELLSTON WATERS, Level 14, NCR House, 342 Lambton Quay, Wellington, New Zealand <b>Primary Examiner:</b> CLAIRE MCINNES <b>Journal:</b> 1461					
<b>Office title:</b> Use of a collagen derivative as an fibroblast activation protein alpha (FAP-alpha) inhibitor <b>(54) Applicant title:</b> Isolated dimeric fibroblast activation protein alpha, and uses thereof					
<b>(57) Abstract:</b>  Patent 335543  Use of an inhibitor of FAP-alpha activity which interacts with molecules having FAP-alpha activity, said inhibitor being a collagen derivative, in the manufacture of a medicament for treating a subject with a pathological condition characterized by abnormal FAP-alpha activity.					

\*\* End of report \*\*

335543

Patents Form No. 5

Our Ref: JP801966

NEW ZEALAND  
PATENTS ACT 1953

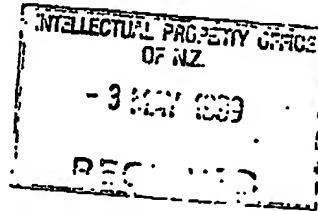
COMPLETE SPECIFICATION

**DIVISIONAL APPLICATION OUT OF  
NEW ZEALAND PATENT APPLICATION NO. 331768  
FILED ON 12 MARCH 1997**

We, **BOEHRINGER INGELHEIM INTERNATIONAL GMBH** a body corporate organised under the laws of Germany of Corporate Patent Department, D-6507, Ingelheim Am Rhein, Fed Republic Of Germany, Fed Republic Of Germany and **LUDWIG INSTITUTE FOR CANCER RESEARCH**, a research institute of 605 Third Avenue, New York, New York 10158, United States of America

hereby declare the invention, for which We pray that a patent may be granted to us and the method by which it is to be performed, to be particularly described in and by the following statement:

PT0540153



335543

PCT/US97/04215

WO 97/34927

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ISOLATED DIMERIC FIBROBLAST ACTIVATION  
PROTEIN ALPHA, AND USES THEREOF

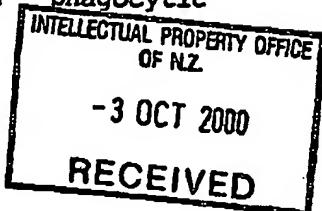
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FIELD OF THE INVENTION

This invention relates to certain molecules associated with cancer tissues and reactive tumor stromal cells. More particularly, it relates to fibroblast activation protein alpha ("FAP $\alpha$ " hereafter) molecules. A monomeric form of the molecule has previously been identified immunochemically, but nucleic acid molecules coding for it had not been isolated or cloned nor have dimers been identified. These, *inter alia*, are features of the invention. The monomeric protein has a molecular weight of from about 88 to about 95 kilodaltons as determined by SDS-PAGE of boiled samples. The dimer has a molecular weight of about 170 kilodaltons as determined by SDS-PAGE of unboiled samples. FAP $\alpha$  is characterized by a number of features and properties which are shared by and characteristic of membrane bound enzymes, suggesting very strongly that it, too, is a membrane bound enzyme. The nucleic acid molecules, which are a key part of the invention, are useful both as probes for cells expressing FAP $\alpha$ , and as starting materials for recombinant production of the protein. The FAP $\alpha$  protein can then be used to produce monoclonal antibodies specific for the protein and are thus useful diagnostic agents themselves. They also have additional uses, including uses related to enzymatic functions, as described herein.

BACKGROUND AND PRIOR ART

The invasive growth of epithelial cancers is associated with characteristic cellular and molecular changes in the supporting stroma. For example, epithelial cancers induce the formation of tumor blood vessels, the recruitment of reactive tumor stromal fibroblasts, lymphoid and phagocytic



5           infiltrates, the release of peptide mediators and proteolytic enzymes, and the production of an altered extracellular matrix (ECM). See, e.g., Folkman, *Adv. Cancer Res.* 43: 175-203 (1985); Bassett et al., *Nature* 348: 699-704 (1990); Denekamp et al., *Cancer Metastasis Rev.* 9: 267-282 (1990); Cullen et al., *Cancer Res.* 51: 4978-4985 (1991); Dvorak et al., *Cancer Cells* 3: 77-85 (1991); Liotta et al., *Cancer Res.* 51: 5054s-5059s (1991); Garin-Chesa et al., *J. Histochem. Cytochem.* 37: 1767-1776 (1989). A highly consistent molecular trait of the stroma in several common histologic types of epithelial cancers is induction of the fibroblast activation protein (FAP $\alpha$ ), a cell surface glycoprotein with an observed  $M_r$  of 95,000 originally discovered with a monoclonal antibody, mAb F19, raised against proliferating cultured fibroblasts. See Rettig et al., *Cancer Res.* 46: 6406-6412 (1986); Rettig et al., *Proc. Natl. Acad. Sci. USA* 85: 3110-3114 (1988); Garin-Chesa et al., *Proc. Natl. Acad. USA* 87: 7235-7239 (1990); Rettig et al., *Cancer Res.* 53: 3327-3335 (1993). Each of these four papers is incorporated by reference in its entirety.

10           Immunohistochemical studies such as those cited supra have shown that FAP $\alpha$  is transiently expressed in certain normal fetal mesenchymal tissues but that normal adult tissues are generally FAP $\alpha$  $^-$ . Similarly, malignant epithelial, neural and hematopoietic cells are generally FAP $\alpha$  $^-$ . However, most of the common types of epithelial cancers, including >90% of breast, lung, skin, pancreas, and colorectal carcinomas, contain abundant FAP $\alpha$  $^+$  reactive stromal fibroblasts. Garin-Chesa et al., *Proc. Natl. Acad. Sci. USA* 87: 7235-7239 (1990). 15           The FAP $\alpha$  $^+$  tumor stromal fibroblasts almost invariably accompany tumor blood vessels, forming a distinct cellular compartment interposed between the tumor capillary endothelium and the basal aspect of malignant epithelial cell clusters. While FAP $\alpha$  $^+$  stromal fibroblasts are found in both primary and 20           metastatic carcinomas, benign and premalignant epithelial lesions, such as fibroadenomas of the breast and colorectal adenomas only rarely contain FAP $\alpha$  $^+$  stromal cells. In contrast 25

to the stroma-specific localization of FAP $\alpha$  in epithelial neoplasms, FAP $\alpha$  is expressed in the malignant cells of a large proportion of bone and soft tissue sarcomas. (Rettig et al., Proc. Natl. Acad. Sci. USA 85: 3110-3114 (1988)). Finally, FAP $\alpha$ \* fibroblasts have been detected in the granulation tissue of healing wounds (Garin-Chesa et al., *supra*). Based on the restricted distribution pattern of FAP $\alpha$  in normal tissues and its uniform expression in the supporting stroma of many epithelial cancers, clinical trials with  $^{131}\text{I}$ -labeled mAb F19 have been initiated in patients with metastatic colon cancer (Welt et al., Proc. Am. Assoc. Cancer Res. 33: 319 (1992); Welt et al. J. Clin. Oncol. 12: 1561-1571 (1994)) to explore the concept of "tumor stromal targeting" for immunodetection and immunotherapy of epithelial cancers.

Rettig et al., Int. J. Cancer 58: 385-392 (1994), incorporated by reference, discusses the FAP $\alpha$  molecule and its features. Rettig et al postulate that FAP $\alpha$  is found in high molecular weight complexes in excess of 400 kilodaltons, but do not discuss the possibility of dimeric molecules, nor does the paper elaborate on the specific enzymatic properties of the molecule.

The induction of FAP $\alpha$ \* fibroblasts at times and sites of tissue remodeling during fetal development, tissue repair, and carcinogenesis is consistent with a fundamental role for this molecule in normal fibroblast physiology. Thus, it is of interest and value to isolate and to clone nucleic acid molecules which code for this molecule. This is one aspect of the invention, which is described in detail together with other features of the invention, in the disclosure which follows. Further aspects of the invention include the dimeric FAP $\alpha$  molecules, and the exploitation of the properties of these molecules. These features are also elaborated upon hereafter.

The applicant's related parent application NZ 331758 provides an isolated, dimeric FAP $\alpha$  molecule, having a molecular weight of about 170 kilodaltons as determined by SDS-PAGE, wherein said dimeric FAP $\alpha$  molecule is capable of degrading extracellular matrix proteins.

(Followed by page 3a)

3a

NZ 331758 also provides a method for cleaving a terminal dipeptide of formula Xaa-Pro from a molecule, comprising contacting said molecule with a second molecule, said second molecule having FAP $\alpha$  enzymatic activity.

NZ 331758 also provides a method for identifying a substance which interacts with a molecule having FAP $\alpha$  activity, comprising combining said molecule with a sample to be tested, and determining any interaction with said molecule as an indication of a molecule which interacts with a molecule having FAP $\alpha$  activity.

NZ 331758 also provides a method for determining if a substance interacts with a molecule having FAP $\alpha$  activity, comprising combining said substance and said molecule with Ala-Pro-AFC, determining interaction of said molecule with Ala-Pro-AFC, and comparing said interaction to interaction of said molecule with Ala-Pro-AFC in the absence of said substance, wherein in a difference therebetween indicate that said substance interacts with said molecule.

NZ 331758 also provides fusion protein comprising a portion of an FAP $\alpha$  molecule sufficient to retain FAP $\alpha$  activity and a non FAP $\alpha$  amino acid sequence, wherein said fusion protein is water soluble.

The present, divisional application provides a method for treating a subject with a pathological condition characterized by abnormal FAP $\alpha$  activity, comprising administering to a subject in need thereof an amount of a substance which interacts with molecules having FAP $\alpha$  activity sufficient to normalize the FAP $\alpha$  activity level in said subject.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 compares the deduced amino acid sequence for FAP $\alpha$ , and the known sequence of CD26. The alignment has been optimized.

5 Figures 2A-2H, inclusive, display immunohistochemical detection of FAP $\alpha$  and CD26 in various tissues. In figures 2A and 2B, breast cancer is studied, for FAP $\alpha$  (figure 2A), and CD26 (figure 2B). In figures 2C and 2D, malignant fibrous histiocytoma is studied, for FAP $\alpha$  (figure 2C), and CD26 (figure 2D). Dermal scar tissue is examined in figures 2E (FAP $\alpha$ ), and 2F (CD26). Renal cell carcinoma is studied in figure 2G (FAP $\alpha$ ), and 2H (CD26).

10 Figure 3 presents some of the data generated in experiments which showed that FAP $\alpha$  had extracellular matrix (ECM) protein degrading activity. When zymographic detection of gelatin degrading extracts of 293-FAP was carried out, the active substance was found to have a molecular weight of about 170 kD, via SDS-PAGE, using unboiled samples to preserve enzyme activity.

15 DETAILED DESCRIPTION OF PREFERRED EMBODIMENTS

Example 1

20 Fibroblast cell line WI-38 had been observed, previously, to react with mAb F19 (Rettig et al., Canc. Res. 46: 6406-6412 (1986); Rettig et al., Proc. Natl. Acad. USA 85: 3110-3114 (1988); Garin-Chesa et al., Proc. Natl. Acad. Sci. USA 87: 7235-7239 (1990); Rettig et al., Canc. Res. 53: 3327-3335 (1993)). It was used in the experiments which follow.

25 A cDNA library was prepared from WI-38, using well known techniques and commercially available materials. Specifically, the library was constructed in expression vector pCDNA1, using the Fast Track mRNA isolation kit, and Librarian cDNA phagemid system. Once the library was prepared, the vectors were electroporated into cell line *E. coli* MC 1061/P3. The pCDNA1 expression vector contains an antibiotic resistance gene, so the *E. coli* were selected via antibiotic resistance. The colonies which were resistant were then used in further experiments. The plasmid DNA from the colonies was obtained via alkaline lysis and purification on CsCl<sub>2</sub>, in accordance 30 with Sambrook et al, Molecular Cloning: A Laboratory Manual (Cold Spring Harbor Lab, Cold Spring Harbor, N.Y. 2d Ed. 1989). The technique is well known to the art, but is

incorporated by reference herein.

Once the plasmid DNA was isolated, it was used to transfect COS-1 cells, which were then cultured for forty-eight hours, after which these were tested with antibody coated dishes. The mAbs used included F19, as described by Rettig et al., (1986), supra, which is incorporated by reference in its entirety. As COS-1 cells are normally FAP $\alpha$ <sup>-</sup>, any positive results indicated the presence of the coding sequence. The immunoselection protocol was that of Aruffo et al., Proc. Natl. Acad. Sci USA 84: 3365-3369 (1987), incorporated by reference herein.

Plasmid DNA from positive clones was recovered, in accordance with Hirt, J. Mol. Biol. 26: 365-369 (1967), reintroduced into *E. coli* MC 1061/P3, and reselected in COS-1 cells.

The protocol presented herein was followed for four rounds. After this, the plasmid DNA of 50 isolated bacterial colonies was purified, using the Qiagen plasmid kit. Of the colonies, 27 clones were found to contain identical 2.8 kb inserts, as determined by EcoRI restriction enzyme mapping. Several of these were found to contain FAP $\alpha$ -specific cDNA as determined by transient expression in COS-1 cells and direct immunofluorescence staining with mAb F19. One of these clones, i.e., "pFAP.38" was selected for further study, as elaborated upon infra.

#### Example 2

Once pFAP.38 had been identified, it was tested together with a vector coding for known cell surface marker CD26 ("pCD26"), as well as with control vector pCDNA I.

In these experiments, COS-1 cells were transfected with one of pFAP.38, pCD26, or pCDNAI. After forty-eight hours, the transfectants were tested, using the well known MHA rosetting assay for cell surface antigen expression. In these experiments, mAb F19, which is FAP $\alpha$  specific, was used, together with mAb EF-1, which is CD26 specific. Also used were four other FAP $\alpha$  specific mAbs, i.e., FB23, FB52, FB58 and C48. Also tested were two cancer cell lines, which are known

to react with mAb F19 (SW872 liposarcoma), or EF-1 (SK-OV6 ovarian cancer). The results are set forth in Table 1, which follows.

5      Table 1. Cell surface expression of multiple PAP $\alpha$  epitopes and CD26 in human cells and COS-1 cell transfectants

Target cell	Cell surface antigen expression					
	F19	FB23	FB52	FB58	C48	EF-1
<u>Human cells</u>						
SW872 liposarcoma	>95%	>95%	>95%	>95%	>95%	-
SK-OV6 ovarian cancer	-	-	-	-	-	>95%
<u>COS-1 transfectants</u>						
COS-pCDNA1 control	-	-	-	-	-	-
COS-pFA P 38	40%	30%	40%	20%	20%	-
COS-pCD26	-	-	-	-	-	40%

35      Example 3

Immunoprecipitation studies were then carried out to identify the antigen being targeted by the antibodies.

40      Cells were metabolically labelled with Trans  $^{35}$ S-label, (ICN), extracted with lysis buffer (0.01 M Tris-HCl/0.15 M NaCl/0.01 M MgCl<sub>2</sub>/0.5% Nonidet P-40/aprotinin (20 ug/ml)/2 mM phenylmethyl-sulfonyl fluoride), and then immunoprecipitated. The protocols used are all well known, as will be seen by reference to Rettig et al., Canc. Res. 53: 3327-3335 (1993); and Fellinger et al., Canc. Res. 51: 336-340 (1991), the disclosures of which are all incorporated by reference in

their entirety. Precipitating mAbs were negative control mouse Ig, mAb F19, or EF-1. Control tests were carried out with mock transfected COS-1 cells. Following immunoprecipitation, the immunoprecipitates were boiled in extraction buffer and separated by NaDODSO<sub>4</sub>/PAGE, under reducing conditions. In some experiments, an additional test was carried out to determine whether or not the immunoprecipitated material was glycosylated. In these experiments, cell extracts were fractionated with Con A-SEPHAROSE prior to immunoprecipitation. Following immunoprecipitation, but prior to fractionation on NaDODSO<sub>4</sub>/PAGE, these precipitates were digested with N-Glycanase.

The results showed that, in COS-1 cells, pFAP.38 directs expression of an 88 kd protein species (as determined via SDS-PAGE), which is slightly smaller than the 95 kd FAP $\alpha$  species produced by SW872, or cultured fibroblasts. Digestion with N-Glycanase produced peptides of comparable size (i.e., 74 kd versus 75 kd), showing that the glycosylation of the FAP $\alpha$  protein in COS-1 cells is different than in the human cell lines.

Example 4

Classic Northern blot analysis was then carried out, using the mRNA from FAP $\alpha$  fibroblast cell lines WI-38 and GM 05389, and FAP $\alpha$ -ovarian cancer cell line SK-OV6. Using the procedures of Sambrook et al., *supra*, five micrograms of mRNA from each cell line were tested. The probes used were <sup>32</sup>P labelled, and were prepared from a 2.3 kb ECO I fragment of pFAP.38, a 2.4 kb Hind III fragment of CD26, and a 1.8 kb BamHI fragment of  $\gamma$ -actin cDNA. These fragments had been purified from 1% agarose gels.

The extracts of FAP $\alpha$  fibroblast strains showed a 2.8 kb FAP mRNA species, but extracts of SK-OV6 do not. A  $\gamma$ -actin mRNA species (1.8 kb), was observed in all species.

Example 5

The cDNA identified as coding for FAP $\alpha$  was subjected to more detailed analysis, starting with sequencing. The classic

Sanger methodology, as set forth in Proc. Natl. Acad. Sci. USA 74: 5463-5467 (1977), was used to sequence both strands of the cDNA. Once this was secured, an amino acid sequence was deduced therefrom. This information is presented in SEQ ID NO: 1. The sequence was then compared to the known amino acid sequence of CD26 (Morimoto et al., J. Immunol. 143: 3430-3437 (1989)). Figure 1 presents the comparison, using optimized sequence alignment. Any gaps in the comparison are indicated by asterisks, while identical amino acids are shown by dashes in the CD26 sequence. A hydrophobic, putative transmembrane sequence is double underlined, while potential N-glycosylation sites are single underlined.

The sequence analysis shows a 2812 base pair insert, wherein 2277 base pairs constitute the open reading frame. This ORF extends from start codon ATG at nucleotide 209, to stop codon TAA at 2486.

The deduced polypeptide is 760 amino acids long, and has a molecular weight of 87,832. In contrast, N-Glycanase digested, immunopurified FAP $\alpha$  was reported to have an estimated M<sub>r</sub> of 75,000 on NaDODSO<sub>4</sub>/PAGE (Rettig et al., Canc. Res. 53: 3327-3335 (1993)). A GenBank data base search was carried out. The most closely related genes found were those encoding dipeptidyl peptidase IV homologues (DPPIV; EC 3.4.14.5), with human DPPIV (also known as T-cell activation antigen CD26), showing 61% nucleotide sequence identity, and 48% amino acid sequence identity.

The second set of related genes are human, rat, and bovine homologues of DPPX, a gene of unknown function widely expressed in brain and other normal tissues. The predicted human DPPX gene product shows about 30% amino acid sequence identity with FAP $\alpha$  and CD26. The FAP $\alpha$  molecule exhibits structural features typical of type II integral membrane proteins, including a large COOH-terminal extracellular domain, a hydrophobic transmembrane segment, and a short cytoplasmic tail. The putative extracellular domain contains five potential N-glycosylation sites, eleven cysteine residues (eight of which are conserved between FAP $\alpha$  and CD26), and

5 three segments corresponding to highly conserved catalytic domains characteristic of serine proteases, such as DPPIV. These conserved sequences are presented in Table 2, which follows. Comparisons to DPPIV and DPPX were made via Morimoto et al., *supra*; Wada et al., Proc. Natl. Acad. Sci. USA 89: 197-201 (1992); Yokotani et al., Human Mol. Genet. 2: 1037-1039 (1993).

10 Example 6

15 An additional set of experiments were carried out to determine whether FAP $\alpha$  related sequences are present in non-human species. To do so, human, mouse, and Chinese hamster genomic DNA was digested using restriction enzymes, and tested, via Southern blotting, using the 2.3 kb fragment, labelled with  $^{32}$ P, described supra. Hybridization was carried out using stringent washing conditions (0.1 x SSC, 0.1% NaDODSO<sub>4</sub>, 68°C). Cross-hybridization was readily observed with both the mouse and hamster DNA, suggesting the existence of highly conserved FAP $\alpha$  homologues. In control experiments using the CD26 cDNA fragment described supra, no evidence of cross hybridization was observed.

20 Example 7

25 The CD26 molecule shares a number of biochemical and serological properties with FAPB, which is a previously described, FAP $\alpha$  associated molecule having a molecular weight of 105 kd, and is found on cultured fibroblasts and melanocytes (Rettig et al., Canc. Res. 53: 3327-3335 (1993)). Cotransfection experiments were carried out to determine whether FAPB is a CD26 gene product. To test this, the same protocols were used which were used for transfection with pFAP.38 or pCD26, as described *supra*, but using the two vectors. The results presented supra showed that cotransfection efficiency was about 40% for each vector, so about 10-20% of cell should be cotransfected.

30 Following cotransfection, the COS-1 cells were Trans  $^{35}$ S-labeled, as described supra, then lysed, also as described supra.

35 The resulting cell extracts were separated on Con A

SEPHAROSE, and the antigen (FAP $\alpha$  and/or CD26) were recovered in the Con A-bound fraction. The bound fraction was eluted with 0.25 M  $\alpha$ -D-mannopyranoside. Immunoprecipitation was then carried out, as described supra, and the precipitates were separated on NaDODSO<sub>4</sub>/PAGE, also as discussed supra.

Those cells transfected only with pFAP.38 produced FAP $\alpha$ , but not FAP $\beta$  (determined from mAb P19 immunoprecipitates). They also produce no CD26 antigen (tested with EF-1). Those cells transfected with pCD26 alone produce CD26 but no FAP $\alpha$ . Cotransfectants produce CD26 and FAP $\alpha$ /FAP $\beta$  heteromers, as determined in the mAb P19 precipitates. This result provides direct evidence that FAP $\beta$  is a CD26 gene product.

Example 8

It has been observed previously that some cultured human cell types coexpress FAP $\alpha$  and CD26, and show FAP $\alpha$ /CD26 heteromer formation. In vivo distribution patterns of FAP $\alpha$  and CD26, however, as determined in previous immunohistochemical studies, appeared to be non-overlapping. (See Rettig et al., Proc. Natl. Acad. Sci. USA 85: 3110-3114 (1988); Garin-Chesa et al., Proc. Natl. Acad. Sci. USA 87: 7235-7329 (1990); Rettig et al., Canc. Res. 53: 3327-3335 (1993); Stein et al., in Knapp et al., eds. Leukocyte typing IV-white cell differentiation antigens, pp 412-415 (Oxford University Press, N.Y. 1989), pp. 412-415; Möbius et al., J. Exp. Immunol. 74: 431-437 (1988)). In view of the potential significance of FAP $\alpha$ /CD26 coassociation, tissue distribution was reexamined, via side by side immunohistochemical staining of normal tissues and lesional tissues known to contain FAP $\alpha^+$  fibroblasts or FAP $\alpha^+$  malignant cells.

To test the samples, they were embedded in OCT compound, frozen in isopentane precooled in liquid nitrogen, and stored at -70°C until used. Five micrometer thick sections were cut, mounted on poly-L-lysine coated slides, air dried, and fixed in cold acetone (4°C, for 10 minutes). The sections were then tested with mAbs (10-20  $\mu$ g/ml), using the well known avidin-biotin immuno-peroxidase method, as described by, e.g., Garin-Chesa et al., J. Histochem. Cytochem. 37: 1767-

## 11

1776 (1989); Garin-Chesa et al., Proc. Natl. Acad. Sci. USA 87: 7235-7239 (1990); Rettig et al., Canc. Res. 53: 3327-3335 (1993); Garin-Chesa et al., Am. J. Pathol. 142: 557-567.

5       The results are shown in figure 2. Breast, colorectal, pancreas and lung carcinomas showed strong expression of FAP $\alpha$  and no CD26 was found (see figures 2A and 2B). Five FAP $\alpha$  sarcomas, including malignant fibrous histiocytoma (figures 2C and 2D), were tested, and there was no expression of CD26. Examination of reactive fibroblasts of healing dermal wounds (figures 2E, 2F), showed abundant expression of both FAP $\alpha$  and CD26. The three renal carcinomas tested (figures 2G, 2H), showed expression of CD26 in malignant epithelium. FAP $\alpha$  was absent from malignant epithelial cells, and showed low expression in the stroma of these carcinomas.

15       Example 9

A mammalian cell line, transfected with a FAP $\alpha$  encoding cDNA, was prepared.

20       Human embryonic kidney cell line 293 is well known and widely available from, e.g., the American Type Culture Collection.

25       Samples of 293 were maintained, in an incubator, at 37°C, in an atmosphere of 95% air, and 5% CO<sub>2</sub>. The cells were cultured in a 50:50 mixture of Dulbecco's modified minimal essential medium and Ham's F12 medium, augmented with 10% fetal bovine serum, penicillin and streptomycin. Following the procedures described by Ustar et al., Eur. Mol. Biol. J. 1991, and/or Park et al., J. Biol. Chem. 169: 25646-25654 (1994), both of which are incorporated by reference, cDNA for FAP $\alpha$  (i.e., SEQ ID NO: 1), was transfected into the 293 cells. Details of the cDNA vector are provided, supra (pFAP.38). Transfectants were selected for resistance to antibiotics (200 ug/ml Geneticin), and were then maintained in selection medium, containing Geneticin.

30       35       Individual colonies of resistant cells were picked, grown to confluence in 6 well tissue culture plates, and were tested for FAP $\alpha$  expression in an immunofluorescence assay (IFA), using FAP $\alpha$  specific monoclonal antibody F19 as described

supra.

Those colonies which expressed FAP $\alpha$  were expanded, and monitored by indirect IFA and cytofluorometric analysis, also as set forth, supra.

The IFAs were positive for the transfectants, referred to hereafter as cell line 293-FAP, but were negative for parental line 293.

Example 10

In order to confirm that recombinant FAP $\alpha$  was, in fact, being produced, a series of immunoprecipitation experiments were carried out. These followed the methods of Park, et al., supra, and Rettig et al., Canc. Res. 53: 3327-3335 (1993), both of which are incorporated by reference. Essentially,  $^{35}$ [S] methionine labelled cell extracts were combined with monoclonal antibody F19, in the manner described supra. Precipitates were then boiled in extraction buffer and run on SDS-PAGE gels, using, as a negative control, mouse IgG1. Both cell line 293-FAP, and non transfected line 293 were tested. The results indicated clearly, that recombinant FAP $\alpha$  was produced by the transfected cell line 293-FAP. This was determined by immunoprecipitation analyses, using FAP $\alpha$  specific monoclonal antibody F19.

Example 11

The ability to produce recombinant FAP $\alpha$  permitted further study of the molecule's properties. Specifically, given the structural features outlined in the prior examples, experiments were designed to determine if FAP $\alpha$  possesses enzymatic activities. The experiments were designed to test whether or not FAP $\alpha$  had extracellular matrix (ECM) protein degrading activity.

Extracts of 293-FAP cells were prepared, using an extraction buffer (0.15M NaCl, 0.05M Tris-HCl, pH 7.4, 10 mM MgCl<sub>2</sub>, 1 percent Triton X-114), were cleared by centrifugation (4,000xg, 10 minutes at 4°C), and phase partitioned at 37°C for 10-20 minutes. This was followed by further centrifugation (4000xg, 20 minutes at 20-25°C). Detergent phases were diluted with buffer (0.15 M NaCl, 0.05 M Tris-HCl).

pH 7.4, 5 mM CaCl<sub>2</sub>, 5 mM MgCl<sub>2</sub>, 0.75% Empigen BB), and separated on concanavalin A-Sepharose following Rettig et al., *supra*. Any concanavalin A bound fractions were eluted with 0.25M methyl- $\alpha$ -D-mannopyranoside in elution buffer (0.15 M NaCl, 0.05 M Tris-HCl, pH 7.4, 5mM CaCl<sub>2</sub>, 5 mM MgCl<sub>2</sub>, 0.1% Triton X-100), mixed with zymography sample buffer (0.25 M Tris-HCl, pH 6.8, 8% SDS, 40% glycerol, 0.01% bromophenol blue), at a 3:1 ratio, and used for further analysis.

Aliquots of sample were loaded onto polyacrylamide gels containing 0.1% of either of gelatin or casein. Electrophoresis was then carried out in a Biorad Mini-Protein II system, at 20 mA constant current for 1.5 - 2 hours, until the bromophenol blue due fronts of samples had reached the lower end of the gel. The gel was removed and incubated for one hour at 20-25°C in a 2.5% aqueous solution of Triton X-100 on a rotary shaker. The Triton X-100 solution was decanted, and replaced with enzyme buffer (0.05M Tris-HCl, pH 7.5, 0.2M NaCl, 5 mM CaCl<sub>2</sub>, 5 mM MgCl<sub>2</sub>, 0.02% Brij 35). The gel was then incubated at 37°C or 41°C, followed by staining or destaining at room temperature. Gels were stained with 0.5% of Coomassie Brilliant Blue G-250 in an aqueous solution of 30% methanol and 10% acetic acid for 15, 30, and 60 minutes, respectively. Subsequently, gels were incubated for 15 minutes in an aqueous solution of 30% CH<sub>3</sub>OH and 5% glycerol, followed by drying between sheets of cellophane.

Gelatinase activity was evaluated in accordance with Kleiner et al., *Anal. Biochem.* 218: 325-329 (1994), incorporated by reference in its entirety. This is a routine assay used to determine whether or not a protease capable of digesting gelatin is present. Labelled molecular weight standard were run on the same gels, under reducing conditions, for molecular weight determinations.

Proteolytic activity for defined amino acid sequence motifs were tested, using a well known membrane overlay assay. See Smith et al, *Histochem. J.* 24(9): 637-647 (1992), incorporated by reference. Substrates were Ala-Pro-7-amino-4-trifluoromethyl coumarin, Gly-Pro-7-amino-4-trifluoromethyl

coumarin, and Lys-Pro-7-amino-4-trifluoromethyl coumarin.

The results of these experiments are depicted, in part, in figure 3. This figure shows zymographic detection of gelatin degrading activity, in the cell extracts. See Kleiner et al., supra. A protein species of approximately 170 kilodaltons, as determined by SDS-PAGE, was observed to have gelatin degrading activity. This species, which was found in the 293-FAP cell line, but not in untransfected 293 cells, is thus identified as FAP $\alpha$ . The molecular weight is consistent with a dimer, i.e., a dimeric FAP $\alpha$  molecule.

The proteolytic activity described herein where gelatin is the substrate, was not observed when casein was the substrate.

#### Example 12

Further studies were then undertaken in order to characterize the 170 kD FAP $\alpha$  dimer further. Specifically, the experiments described in example 11 were repeated, except that 5% of 2-mercaptoethanol or 5  $\mu$ M iodoacetamide was added to the extracts prior to SDS-PAGE, or ethylenediamine N,N,N',N'-tetraacetic acid (10 mM) was added to the incubation buffer used for gelatin zymography. None of these treatments abolished the enzymatic activity. In contrast, heating at 100°C for five minutes prior to SDS-polyacrylamide gel electrophoresis abolished the gelatin-degrading activity.

Further work, using a membrane overlay assay, described by, e.g., Smith et al., Histochem J. 24(9): 643-647 (1992), incorporated by reference, revealed that the FAP $\alpha$  dimers were able to cleave all of the Ala-Pro, Gly-Pro, and Lys-Pro dipeptides tested.

In further experiments, a fusion protein was produced which comprised the extracellular domains of both FAP $\alpha$  and murine CD8 proteins. This chimeric protein was produced in a baculovirus system in insect cells. The chimeric protein exhibited the same enzymatic activity as FAP $\alpha$ , using the model discussed *supra*.

#### Example 13

Two quantitative assays for FAP $\alpha$  enzyme activity were developed using Ala-Pro-7-amino-4-trifluoromethyl coumarin (Ala-Pro-AFC) as the substrate. In the first assay format, membrane extracts of FAP $\alpha$ -expressing cells were mixed with a 5-10 fold volume of reaction buffer (100mM NaCl, 100mM Tris pH 7.8), and added to an equal volume of 0.5mM Ala-Pro-AFC in reaction buffer followed by an incubation for one hour at 37°C. Release of free AFC was then measured in a fluorimeter using a 395nm excitation / 530nm emission filter set. The membrane extracts analyzed in this assay format were derived from either 293-FAP $\alpha$  cells (293 cells stably transfected with vector FAP.38 described *supra*) or HT1080-FAP $\alpha$  cells (HT1080 cells stably transfected with vector FAP.38). Negative control experiments assessing FAP $\alpha$ -specific activities were carried out with membrane extracts prepared from the respective parental 293 or HT1080 cell lines. In the second assay, FAP $\alpha$  was isolated from 293-FAP $\alpha$  or HT1080-FAP $\alpha$  membrane extracts via an antibody specific for FAP $\alpha$ . Ninety-six well ELISA plates were coated overnight at 4°C with 1ug/ml F19 monoclonal antibody in phosphate-buffered saline (PBS). In the case of CD8-FAP $\alpha$  discussed *infra* plates were coated with F19 antibody as above or with 1ug/ml rat anti-mouse CD8 overnight at 4°C. Wells were then washed with wash buffer (PBS, 0.1% Tween 20). Excess binding sites were blocked with blocking buffer (5% bovine serum albumin in PBS) for 1 hour at room temperature. Blocking buffer was removed; membrane extracts of 293-FAP $\alpha$  expressing cells or control cells were added and incubated for 1 hour at room temperature. The unbound material was removed, wells were washed with wash buffer, and FAP $\alpha$  activity was assayed using 100 ul Ala-Pro-AFC (0.5 mM Ala-Pro-AFC in reaction buffer) for one hour at 37°C. Release of free AFC was measured as above. Binding of mab F19 to FAP $\alpha$  did not measurably affect its enzymatic activity.

Example 14

Using assays for FAP $\alpha$  enzyme activity, described *supra* an inhibitor of FAP $\alpha$  enzymatic activity has been identified. This inhibitor is (S)-Valylpyrrolidine-2(R)-boronic acid (Snow

et al., J. Am. Chem Soc.(1994) 116:10860-10869), referred to here as ValboroPro. ValboroPro inhibits cleavage of Ala-Pro-AFC by FAP $\alpha$  with an IC<sub>50</sub> of 0.11 uM. ValboroPro also inhibits the gelatinolytic activity of FAP $\alpha$  at a concentration of 100 uM. The specificity of ValboroPro for FAP $\alpha$  was demonstrated in tests with an unrelated serine protease, trypsin. No inhibition of bovine trypsin by ValboroPro (up to 100 uM) was observed when assayed with carbobenzoxy-L-valinyl-glycyl-L-arginyl-4-nitranilide acetate as substrate.

Example 15

The identification of specific, structural requirements for the enzymatic activities of FAP $\alpha$  facilitates the development of molecules which can bind to and/or inhibit FAP $\alpha$ . To examine whether the serine residue at position 624 of the predicted amino acid sequence of FAP $\alpha$  polypeptide is critical for its enzymatic function, site-directed mutagenesis according to Zoller, et al DNA 3:479-488 (1984) was performed using standard polymerase chain reaction methods. The TCC codon coding for serine 624 in the FAP $\alpha$  cDNA was replaced with GCG, resulting in alanine at this position. The altered DNA was reintroduced into the FAP.38 vector and transfected into 293 cells as described *supra*. Geneticin-resistant colonies were selected and examined by indirect IFA for FAP $\alpha$  expression using mAb P19 as well as other FAP $\alpha$  specific antibodies described by Rettig, et al., J. Cancer 58:385-392 (1994) as set forth, *supra*. No differences in binding of the anti-FAP $\alpha$  antibodies to the mutant FAP $\alpha$  expressing cells were observed as compared to wild type FAP $\alpha$  transfected cells. The presence of the mutation was confirmed through amplification of genomic DNA and restriction enzyme digestion performed with several clones of transfected cells. To assess the enzymatic activity of mutant FAP $\alpha$ , the following tests were performed. Membrane extracts were prepared from three independent positive clones and equal amounts of FAP $\alpha$  protein (as determined in a double-determinate ELISA assay using two anti-FAP $\alpha$  antibodies that recognize distinct FAP $\alpha$  epitopes) were examined in the gelatinolytic and Ala-Pro-AFC capture assays.

Both the gelatinolytic activity and the activity in the capture assay of isolated mutant FAP $\alpha$  were reduced to undetectable levels compared to wild type FAP $\alpha$ , confirming the role of the canonical serine in the catalytic triad for both observed enzymatic activities.

Example 16

A fusion protein was generated to obtain secreted, water-soluble FAP $\alpha$  enzyme. In this fusion protein, the extracellular domain of CD8, consisting of the first 189 amino acids of murine CD8, was linked to the extracellular domain of FAP $\alpha$  (amino acids 27 to 760), as described by Lane et al., J. Exp. Med. 177:1209 (1993) using standard polymerase chain reaction protocols and inserted in commercially available pVL1393 vector. Transfection of Sf9 cells with this vector and amplification of the resulting recombinant baculovirus were performed as described (Baculovirus Expression Vectors, O'Reilly, Miller, and Luckow, Oxford University Press, 1994). The CD8-FAP fusion protein was isolated in a two step purification from the spent medium of High Five<sup>TM</sup> cells infected with CD8-FAP $\alpha$  baculovirus for four days. Cells and virus were removed by ultracentrifugation, the supernatant was passed through a column containing Heparin-Sepharose (Pharmacia) and eluted stepwise with 0.6, 1.0, and 2.0 M NaCl in 10mM phosphate, pH 7. Active fractions from the 1.0 and 2.0 M eluates were pooled and concentrated using an YM-10 filter and 26/60 Superdex-200 gel filtration column. Activity was observed in a high molecular weight peak which, when subjected to N-terminal gas phase sequencing, was confirmed to be CD8-FAP $\alpha$ . In gelatinolytic assays, activity greater than 200kD in the gelatinolytic assay was detected when purified CD8-FAP $\alpha$  was tested, consistent with the higher predicted molecular weight of the fusion protein.

Example 17

The presence of structural and functional homologues in non-human species has been ascertained. For example, the cDNA for mouse FAP $\alpha$  has been cloned and characterized. Examination of the predicted amino acid sequence of the homologous mouse

FAP $\alpha$  cDNA sequence (EMBL accession number Y10007) reveals a high degree of conservation of FAP $\alpha$  across species. The two proteins are 89% identical and the catalytic triad is conserved between human FAP $\alpha$  and mouse FAP $\alpha$ . The high degree of conservation and similar tissue expression suggests that FAP $\alpha$  from nonhuman sources may be functionally equivalent to human FAP $\alpha$ . This conclusion is confirmed by the finding that a CD8-murine FAP $\alpha$  fusion protein similar in design to CD8-human FAP $\alpha$  also demonstrates the expected dipeptidylpeptidase enzymatic activity using Ala-Pro-AFC as substrate.

The foregoing examples describe an isolated nucleic acid molecule which codes for fibroblast activating protein alpha ("FAP $\alpha$ "), as well as dimeric forms of the molecule, and uses thereof. The expression product of the sequence in COS-1 is a protein which, on SDS-PAGE of boiled samples, shows a molecular weight of about 88 kd. Deduced amino acid sequence, as provided in SEQ ID NO: 1, for one form of the molecule, yields a molecular weight of about 88 kd.

It should be noted that there is an apparent discrepancy in molecular weight in that the COS-1 isolate is glycosylated, while molecular weight from deduced amino acid sequences does not account for glycosylation. Membrane proteins are known to exhibit aberrant migration in gel systems, however, which may explain the difference observed here.

Also a part of the invention are chimeric and fusion proteins, which comprise a portion of FAP $\alpha$  which contain the molecule's catalytic domain, and additional, non FAP $\alpha$  components. The FAP $\alpha$  catalytic domain *per se* is also a part of the invention.

It is to be understood that, as described, FAP $\alpha$  may be glycosylated, with the type and amount of glycosylation varying, depending upon the type of cell expressing the molecule. The experiment described herein shows this. This is also true for the dimeric form of the molecule, first described herein, having a molecular weight of about 170 kilodaltons as determined by SDS-PAGE of unboiled samples.

The invention also comprehends the production of

expression vectors useful in producing the FAP $\alpha$  molecule. In their broadest aspect, these vectors comprise the entire FAP $\alpha$  coding sequence or portions thereof, operably linked to a promoter. Additional elements may be a part of the expression vector, such as protein domains fused to the FAP $\alpha$  protein or protein portions ("fusion protein") genes which confer antibiotic resistance, amplifiable genes, and so forth.

The coding sequences and vectors may also be used to prepare cell lines, wherein the coding sequence or expression vector is used to transfect or to transform a recipient host. The type of cell used may be prokaryotic, such as *E. coli*, or eukaryotes, such as yeast, CHO, COS, or other cell types.

The identification of nucleic acid molecules such as that set forth in SEQ ID NO: 1 also enables the artisan to identify and to isolate those nucleic acid molecules which hybridize to it under stringent conditions. "Stringent condition" as used herein, refers to those parameters set forth supra, whereby both murine and hamster sequences were also identified. It will be recognized by the skilled artisan that these conditions afford a degree of stringency which can be achieved using parameters which vary from those recited. Such variance is apprehended by the expression "stringent conditions".

The ability of nucleic acid molecules to hybridize to complementary molecules also enables the artisan to identify cells which express FAP $\alpha$ , via the use of a nucleic acid hybridization assay. One may use the sequences described in the invention to hybridize to complementary sequences, and thus identify them. In this way, one can target mRNA, e.g., which is present in any cell expressing the FAP $\alpha$  molecule.

It is of course understood that the nucleic acid molecules of the invention are also useful in the production of recombinant FAP $\alpha$ , in both monomeric and dimeric form. The examples clearly show that host cells are capable of assembling the dimeric forms. The recombinant protein may be used, e.g., as a source of an immunogen for generation of antibodies akin to known mAb F19, and with the same uses. Similarly, the recombinant protein, and/or cells which express

the molecule on their surface, may be used in assays to determine antagonists, agonists, or other molecules which interact with molecules having FAP $\alpha$  activity. Such substances may be, but are not necessarily limited to, substrates, inhibiting molecules, antibodies, and so forth. The molecules having FAP $\alpha$  activity may be, e.g., the monomeric or dimeric forms of FAP $\alpha$ , derivatives containing the catalytic domain, and so forth. The molecule having FAP $\alpha$  activity may be pure, or in the form of a cell extract, such as a transformed or transfected cell, which has received an FAP $\alpha$  active gene. Both prokaryotes and eukaryotes may be used. This last feature of the invention should be considered in light of the observed structural resemblances to membrane bound enzymes. This type of molecule is associated with certain properties which need not be described in detail here. It will suffice to say that inhibition or potentiation of these properties as associated with FAP $\alpha$  is a feature of this invention. For example, one may identify substrates or the substrate for FAP $\alpha$  molecules, via the use of recombinant cells or recombinant FAP $\alpha$  per se. The substrates can be modified to improve their effect, to lessen their effect, or simply to label them with detectable signals so that they can be used, e.g., to identify cells which express FAP $\alpha$ . Study of the interaction of substrate and FAP $\alpha$ , as well as that between FAP $\alpha$  and any molecule whatsoever, can be used to develop and/or to identify agonists and antagonists of the FAP $\alpha$  molecule.

Also a feature of the invention are isolated, dimeric FAP $\alpha$  molecules which have a molecular weight of about 170 kilodaltons as determined by SDS-PAGE, their use as an enzymatic cleaving agent, and other uses as are described herein. Enzymatically active forms of FAP $\alpha$  may also be produced as recombinant fusion proteins, such as soluble fusion proteins comprising the catalytic domain of FAP $\alpha$  and other protein domains with suitable biochemical properties, including secretory signals, protease cleavage sites, tags for purification, and other elements known to the artisan.

Exemplary are CDS peptide sequences, such as are described supra. The fact that FAP $\alpha$  has particular properties, as described herein, permits the identification of the molecule on cells expressing them. In turn, because the FAP $\alpha$  molecule is associated with tumors and tumor stromal cells, targeting of FAP $\alpha$  with therapeutic agents serves as a way to treat cancerous or precancerous condition, by administering sufficient therapeutic agent to alleviate cancer load.

The experiments showing the proteolytic properties of FAP $\alpha$  lead to yet a further aspect of the invention. It is well known that proteases which degrade extracellular matrix, or "ECM" proteins have an important role on certain aspects of tumor growth, including their effect on tumor cell invasion, tumor blood vessel formation (i.e., neoangiogenesis), and tumor metastasis. Collagens are of special interest vis-a-vis the substrates of proteases, as the collagens are an important part of the ECM. The fact that FAP $\alpha$  digests ECM suggests a therapeutic role for inhibitors of the molecule. "Inhibitors", as used herein, refers to molecules which interfere with FAP $\alpha$  enzyme function. Specifically excluded from such inhibitors is the monoclonal antibody F19. This mAb is known to bind to but not inhibit the enzyme function of FAP $\alpha$ , and hence it is not an inhibitor. The art is quite well versed with respect to monoclonal antibodies which both bind to and inhibit enzymes. Further examples of such inhibitors would include, e.g., substrate derivatives, such as modified collagen molecules, which interfere with the active site or sites of the FAP $\alpha$  molecule. Other suitable inhibitors will be apparent to the skilled artisan, and need not be listed here. In addition, the recombinant FAP $\alpha$  proteins and FAP $\alpha$ -transfected cell lines described supra can be employed in an enzymatic screening assay, using the substrate described supra or other suitable substrates, to identify inhibitors from any compound library. The identification of substances which interact with FAP $\alpha$  active molecules thereby leads to therapeutic treatment of conditions where a subject exhibit abnormal FAP $\alpha$  activity. Specifically, an amount of an

appropriate substance, be it an inhibitor (e.g., a collagen derivative, S-Valyl-pyrrolidine-2(R)-boronic acid), an agonist or an antagonist is administered to a subject in an amount sufficient to normalize FAP<sub>G</sub> activity.

Other aspects of the invention will be clear to the skilled artisan, and need not be set forth here.

The terms and expressions which have been employed are used as terms of description and not of limitation, and there is no intention in the use of such terms and expressions of excluding any equivalents of the features shown and described or portions thereof, it being recognized that various modifications are possible within the scope of the invention.

## (1) GENERAL INFORMATION:

- (i) APPLICANTS: Zimmermann, Rainer; Park, John E.;  
Rettig, Wolfgang; Old, Lloyd J.
- (ii) TITLE OF INVENTION: ISOLATED DIMERIC FIBROBLAST ACTIVATION PROTEIN ALPHA, AND USES THEREOF
- (iii) NUMBER OF SEQUENCES: 2
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- (B) REGISTRATION NUMBER: 30,946
- (C) REFERENCE/DOCKET NUMBER: LUD 5330.1-PCT

## (x) TELECOMMUNICATION INFORMATION:

- (A) TELEPHONE: (212) 688-9200
- (B) TELEFAX: (212) 838-3884

## (2) INFORMATION FOR SEQ ID NO: 1:

## (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 2815 Base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: double  
(D) TOPOLOGY: linear

## (xi) SEQUENCE DESCRIPTION: SEQ. ID NO: 1:

AAGAACGCC CCAAAATCTG TTTCTAATT TACAGAAATC TTTTGAAC TGGCACGGTA 60  
TTCAAAAGTC CGTGGAAAGA AAAAAACCTT GTCCTGGCTT CAGCTTCCAA CTACAAAGAC 120  
AGACTTGGTC CTTTCAACG GTTTACAG ATCCAGTGAC CCACGCTCTG AAGACAGAAAT 180  
TAGCTAACCTT TCAAAAACAT CTGGAAAAAT GAAGACTTGG GTAAAAATCG TATTTGGAGT 240  
TGCCACCTCT GCTGTGCTTG CCTTATTGGT GATGTGCATT GTCTTACGCC CTTCAAGAGT 300  
TCATAACTCT GAAGAAAATA CAATGAGAGC ACTCACACTG AAGGATATT TAAATGGAAC 360  
ATTTCTTAT AAAACATT TTCCAAACTG GATTCAGGA CAAGAATATC TTCACTCAATC 420  
TGCAGATAAC AATATAGTAC TTATAATAT TGAAACAGGA CAATCATATA CCATTTTGAG 480  
TAATAGAACC ATGAAAAGTG TGAATGCTTC AAATTACGGC TTATCACCTG ATCGGCAATT 540  
TGTATATCTA GAAAGTGATT ATTCAAAGCT TTGGAGATAC TCTTACAGC CAACATATTA 600  
CATCTATGAC CTTAGCAATG GAGAATTGTT AAGAGGAAAT GAGCTTCCTC GTCCAATTCA 660  
GTATTTATGC TGGTCGCTG TTGGGAGTAA ATTAGCATAT GTCTATCAAA ACAATATCTA 720  
TTTGAAACAA AGACCAGGAG ATCCACCTT TCAAATAACA TTTAATGGAA GAGAAAATAA 780  
AATATTTAAT GGAATCCCAG ACTGGGTTA TGAAGAGGAA ATGCTTCCTA CAAAATATGC 840  
TCTCTGGTGG TCTCTTAATG GAAAATTGTT GGCAATATGCG GAATTTAATG ATAAGGATAT 900  
ACCAAGTTATT GCCTATTCCCT ATTATGGCGA TGAACAATAT CCTAGAACAA TAAATATTCC 960  
ATACCCAAAG GCTGGAGCTA AGAATCCCGT TGTTGGATA TTTATTATCG ATACCACTTA 1020  
CCCTCGTAT GTAGGTCCCC AGGAAGTGC TGTTCCAGCA ATGATAGCCT CAAGTGATTA 1080  
TTATTCAGT TGGCTCACGT GGGTTACTGA TGAACGAGTA TGTTTGAGT GGCTAAAAAG 1140  
AGTCCAGAAT GTTTCGGTCC TGTCTATATG TGACTTCAGG GAAGACTGGC AGACATGGGA 1200  
TTGTCCTAAAG ACCCAGGAGC ATATAGAAGA AAGCAGAACT GGATGGGCTG GTGGATTCTT 1260  
TGTTTCAAGA CCAGTTTCA GCTATGATGC CATTCTGTAC TACAAAATAT TTAGTGACAA 1320  
GGATGGCTAC AAACATATTC ACTATATCAA AGACACTGTG GAAAATGCTA TTCAAATTAC 1380  
AAGTGGCAAG TGGGAGGCCA TAAATATATT CAGAGTAACA CAGGATTAC TGTGTTATTC 1440  
TAGCAATGAA TTGGAAGAAT ACCCTGGAAG AAGAAACATC TACAGAATTA GCATTGGAAAG 1500  
CTATCCTCCA AGCAAGAAGT GTGTTACTTG CCATCTAAGG AAAGAAAAGT GCCAATATTA 1560  
CACAGCAAGT TTCAGCGACT ACGCCAAGTA CTATGCACTT GTCTGCTACG GCCCAGGCAT 1620  
CCCCATTTCAC ACCCTTCATG ATGGACGCAC TGATCAAGAA ATTAAAATCC TGGAAGAAAA 1680

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CAAGGAATTG GAAAATGCTT TGAAAAATAT CCAGCTGCCT AAAGAGGAAA TTAAGAAACT 1740  
 TGAAGTAGAT GAAATTACTT TATGGTACAA GATGATTCTT CCTCCTCAAT TTGACAGATC 1800  
 AAAGAAGTAT CCCTTGCTAA TTCAAGTGTA TGGTGGTCCC TGAGTCAGA GTGTAAGGTC 1860  
 5 TGTATTTGCT GTTAATTGGA TATCTTATCT TGCAAGTAAG GAAGGGATGG TCATTGCCTT 1920  
 GGTGGATGGT CGAGGAACAG CTTCCAAGG TGACAAAATC CTCTATGCAG TGTATCGAAA 1980  
 GCTGGGTGTT TATGAAGTTG AAGACCAGAT TACAGCTGTC AGAAAATTCA TAGAAATGGG 2040  
 TTTCATTGAT GAAAAAAAGAA TAGCCATATG GGGCTGGTCC TATGGAGGAT ACCTTTCATC 2100  
 ACTGGCCCTT GCATCTGGAA CTGGTCTTTT CAAATGTGGT ATAGCAGTGG CTCCAGTCTC 2160  
 10 CAGCTGGGAA TATTACGCGT CTGTCTACAC AGAGAGATTG ATGGGTCTCC CAACAAAGGA 2220  
 TGATAATCTT GAGCACTATA AGAATTCAAC TGTGATGGCA AGAGCAGAAT ATTTCAGAAA 2280  
 TGTAGACTAT CTTCTCATCC ACAGGAACAGC AGATGATAAT GTGCACTTTC AAAACTCAGC 2340  
 ACAGATTGCT AAAGCTCTGG TTAATGCACA AGTGGATTTC CAGGCAATGT GGTACTCTGA 2400  
 CCAGAACAC GGCTTATCCG GCCTGTCCAC GAACCACTTA TACACCCACA TGACCCACTT 2460  
 CCTAAACAG TGTTTCTCTT TGTCAGACTA AAAACGATGC AGATGCAAGC CTGATATCAGA 2520  
 15 ATCTGAAAAC CTTATATAAA CCCCTCAGAC AGTTTGCTTA TTTTATTTT TATGTTGTAA 2580  
 AATGCTAGTA TAAACAAACA AATTATGTT GTTCTAAAGG CTGTTAAAAA AAAGATGAGG 2640  
 ACTCAGAACT TCAAGCTAAA TATTGTTAC ATTTCTGGT ACTCTGTGAA AGAAGAGAAA 2700  
 AGGGAGTCAT GCATTTGCT TTGGACACAG TGTTTATCA CCTGTTCATT TGAAGAAAAA 2760  
 TAATAAAAGTC AGAAGTTCAA AAAAAAAAAA AAAAAAAAAA AAAGCGGCCG CTCGA 2815

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## (2) INFORMATION FOR SEQ ID NO: 2:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 760 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

30

Met Lys Thr Trp Val Lys Ile Val Phe Gly Val Ala Thr Ser Ala Val		
5	10	15
Leu Ala Leu Leu Val Met Cys Ile Val Leu Arg Pro Ser Arg Val His		
20	25	30
Asn Ser Glu Glu Asn Thr Met Arg Ala Leu Thr Leu Lys Asp Ile Leu		
35	40	45
Asn Gly Thr Phe Ser Tyr Lys Thr Phe Phe Pro Asn Trp Ile Ser Gly		

35

	50	55	60	
	Gln Glu Tyr Leu His Gln Ser Ala Asp Asn Asn Ile Val Leu Tyr Asn			
5	65	70	75	80
	Ile Glu Thr Gly Gln Ser Tyr Thr Ile Leu Ser Asn Arg Thr Met Lys			
	85	90	95	
	Ser Val Asn Ala Ser Asn Tyr Gly Leu Ser Pro Asp Arg Gln Phe Val			
	100	105	110	
10	Tyr Leu Glu Ser Asp Tyr Ser Lys Leu Trp Arg Tyr Ser Tyr Thr Ala			
	115	120	125	
	Thr Tyr Tyr Ile Tyr Asp Leu Ser Asn Gly Glu Phe Val Arg Gly Asn			
	130	135	140	
	Glu Leu Pro Arg Pro Ile Gln Tyr Leu Cys Trp Ser Pro Val Gly Ser			
	145	150	155	160
	Lys Leu Ala Tyr Val Tyr Gln Asn Asn Ile Tyr Leu Lys Gln Arg Pro			
15	165	170	175	
	Gly Asp Pro Pro Phe Gln Ile Thr Phe Asn Gly Arg Glu Asn Lys Ile			
	180	185	190	
	Phe Asn Gly Ile Pro Asp Trp Val Tyr Glu Glu Glu Met Leu Pro Thr			
	195	200	205	
20	Lys Tyr Ala Leu Trp Trp Ser Pro Asn Gly Lys Phe Leu Ala Tyr Ala			
	210	215	220	
	Glu Phe Asn Asp Lys Asp Ile Pro Val Ile Ala Tyr Ser Tyr Tyr Gly			
	225	230	235	240
	Asp Glu Gln Tyr Pro Arg Thr Ile Asn Ile Pro Tyr Pro Lys Ala Gly			
25	245	250	255	
	Ala Lys Asn Pro Val Val Arg Ile Phe Ile Ile Asp Thr Thr Tyr Pro			
	260	265	270	
	Ala Tyr Val Gly Pro Gln Glu Val Pro Val Pro Ala Met Ile Ala Ser			
	275	280	285	
30	Ser Asp Tyr Tyr Phe Ser Trp Leu Thr Trp Val Thr Asp Glu Arg Val			
	290	295	300	
	Cys Leu Gln Trp Leu Lys Arg Val Gln Asn Val Ser Val Leu Ser Ile			
	305	310	315	320
	Cys Asp Phe Arg Glu Asp Trp Gln Thr Trp Asp Cys Pro Lys Thr Gln			
35	325	330	335	
	Glu His Ile Glu Glu Ser Arg Thr Gly Trp Ala Gly Gly Phe Phe Val			
	340	345	350	

Ser Arg Pro Val Phe Ser Tyr Asp Ala Ile Ser Tyr Tyr Lys Ile Phe  
 355                   360                   365  
 Ser Asp Lys Asp Gly Tyr Lys His Ile His Tyr Ile Lys Asp Thr Val  
 370                   375                   380  
 5 Glu Asn Ala Ile Gln Ile Thr Ser Gly Lys Trp Glu Ala Ile Asn Ile  
 385                   390                   395                   400  
 Phe Arg Val Thr Gln Asp Ser Leu Phe Tyr Ser Ser Asn Glu Phe Glu  
 405                   410                   415  
 Glu Tyr Pro Gly Arg Arg Asn Ile Tyr Arg Ile Ser Ile Gly Ser Tyr  
 10                   420                   425                   430  
 Pro Pro Ser Lys Lys Cys Val Thr Cys His Leu Arg Lys Glu Arg Cys  
 435                   440                   445  
 Gln Tyr Tyr Thr Ala Ser Phe Ser Asp Tyr Ala Lys Tyr Tyr Ala Leu  
 15                   450                   455                   460  
 Val Cys Tyr Gly Pro Gly Ile Pro Ile Ser Thr Leu His Asp Gly Arg  
 465                   470                   475                   480  
 Thr Asp Gln Glu Ile Lys Ile Leu Glu Glu Asn Lys Glu Leu Glu Asn  
 20                   485                   490                   495  
 Ala Leu Lys Asn Ile Gln Leu Pro Lys Glu Glu Ile Lys Lys Leu Glu  
 500                   505                   510  
 Val Asp Glu Ile Thr Leu Trp Tyr Lys Met Ile Leu Pro Pro Gln Phe  
 25                   515                   520                   525  
 Asp Arg Ser Lys Lys Tyr Pro Leu Leu Ile Gln Val Tyr Gly Gly Pro  
 530                   535                   540  
 Cys Ser Gln Ser Val Arg Ser Val Phe Ala Val Asn Trp Ile Ser Tyr  
 30                   545                   550                   555                   560  
 Leu Ala Ser Lys Glu Gly Met Val Ile Ala Leu Val Asp Gly Arg Gly  
 565                   570                   575  
 Thr Ala Phe Gln Gly Asp Lys Leu Leu Tyr Ala Val Tyr Arg Lys Leu  
 580                   585                   590  
 Gly Val Tyr Glu Val Glu Asp Gln Ile Thr Ala Val Arg Lys Phe Ile  
 35                   595                   600                   605  
 Glu Met Gly Phe Ile Asp Glu Lys Arg Ile Ala Ile Trp Gly Trp Ser  
 610                   615                   620  
 Tyr Gly Gly Tyr Val Ser Ser Leu Ala Leu Ala Ser Gly Thr Gly Leu  
 625                   630                   635                   640  
 Phe Lys Cys Gly Ile Ala Val Ala Pro Val Ser Ser Trp Glu Tyr Tyr

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Ala Ser Val Tyr Thr Glu Arg Phe Met Gly Leu Pro Thr Lys Asp Asp

660

665

670

Asn Leu Glu His Tyr Lys Asn Ser Thr Val Met Ala Arg Ala Glu Tyr

675

680

685

Phe Arg Asn Val Asp Tyr Leu Leu Ile His Gly Thr Ala Asp Asp Asn

690

695

700

Val His Phe Gln Asn Ser Ala Gln Ile Ala Lys Ala Leu Val Asn Ala

705

710

715

720

Gln Val Asp Phe Gln Ala Met Trp Tyr Ser Asp Gln Asn His Gly Leu

725

730

735

Ser Gly Leu Ser Thr Asn His Leu Tyr Thr His Met Thr His Phe Leu

740

745

750

Lys Gln Cys Phe Ser Leu Ser Asp

755

760

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WE CLAIM:

1. Use of an inhibitor of FAP $\alpha$  activity which interacts with molecules having FAP $\alpha$  activity, said inhibitor being a collagen derivative, in the manufacture of a medicament for treating a subject with a pathological condition characterised by abnormal FAP $\alpha$  activity.
2. Use according to claim 1, wherein said inhibitor is (S) - Valyl-pyrrolidine-2(R) - boronic acid.
3. Use according to claim 1 and substantially as herein described with reference to any one of the examples.



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## FIG. 1

FAP	1	MKTWVKIVFGV*ATSAVLALLVMCIVLRPSRVHNSEENTMRALTLKDILN	49
CD26	1	---PW-VLL-LLGAA-LVTIITVPV--LNKGTDODATADSRKTY--T-Y-K	50
FAP	50	GTFSYKTFFPNWISGQEYLHQSAADNNIVLYNIETGQSYTILSNRTMKSV*	98
CD26	51	N-YRL-LYSLR---DH---YKQ*E---LVF-A-Y-N-SVF-E-S-FDEFG	99
FAP	99	*NASNYGLSPDRQFVYLEDYSKLWRYSYTATYYIYDLSNGEFVRGNELP	147
CD26	100	ESIND-SI---G--IL--YN-V-Q--H---S-D---NKRQLITEERI-	149
		<u>fab-1</u>	
FAP	148	RPIQYLCWSVGSKLAYVYQNNIYLKQRPGDPFFQITFNGRENKIFNGIP	197
CD26	150	NNT-WVT----E----WN-D-V-IE-NL-SYR--WT-K-DI-Y---T	199
		<u>fab-2</u>	
FAP	198	DWVYEEEMLPTKYALWWSPNGKELAYAEFNNDKIDPVIAYSYYGDE**QYP	245
CD26	200	-----VFSAYS-----T-----Q---TEV-L-E--F-S--SL---	249
FAP	246	RTINIPYPKAGAKNPVVRIFIIDT***TYPAYVGPQEVPVPAMIASSDYY	292
CD26	250	K-VRV-----V--T-KF-VVN-DSLSSVTNATSIQITA--SMLIG-H-	299
FAP	293	FSWLTWVTDERVCLQWLKRVONVSVLICDFREDWQTWDCPKTQEHIIES	342
CD26	300	LCDV--A-Q--IS---R-I--Y--MD---YD-SSGR-N-LVARQ---M-	349
FAP	343	RTGWAGGFVSRPVFSYDAISYYKIFSDKDGYKHIHYIKDTVENAIQITS	392
CD26	350	T---V-R-RP-E-H-TL-GN-F---I-NEE--R--C-FQIDKKDCTF--K	399
FAP	393	GKWEAINIFRVTQDSLFYSSNEFEYPGRRNIYRISIGSYPPSKKCVTCH	442
CD26	400	-T---V-G-EAL-S-Y-Y-I---YKGM--G--L-K-QLSD-T*KVT-LS-E	448
FAP	443	LRKERCQYYTASFSDYAKYYALVCYGPPIPSTLHDGRTDQEIRILEENK	492
CD26	449	-NP-----SV---KE---Q-R-S---L-LY---SSVN-KGLRV--D-S	498
		<u>fab-3</u>	
FAP	493	ELENALKNIQLPKEEIKKLEVDEITLWYKMLPPQFDRSKYPLLIQVYG	542
CD26	499	A-DKM-Q-V-M-SKKLDIILN-TKF--Q-----H--K-----LD--A	548
FAP	543	GPCSQSVRSVFAVNWIYLASKEGMVIALVDGRGTAFQGDKLLYAVYRKL	592
CD26	549	-----KADT--RL--AT----T-NIIV-SF----SGY---IMH-IN-R-	598
FAP	593	GVYEVEDQITAVRKFIEMGFIDEKRRIAIWGWSYEIRFITGPCIWNWSFQM	642
CD26	599	-TF-----E-A-Q-SK---V-N-----GGYVTSMLGSVGFK	648
FAP	643	WYSSGSSLQLGILRVCLHRE*IHGSPNKDDNLHYKNSTVMARAEYFRNV	691
CD26	649	CGIAVAPVSRWEYYDSVT-RYM-L-TPE---D--R-----S---N-KQ-	698
FAP	692	DYLLIINGTADDNVHFQNSAQIAKALVNAQVDFQAMWYSDQNHGLSGLSTN	741
CD26	699	E-----Q---S---DVG-----T-ED--IASSTAH	748
FAP	742	*HLYTHMTHFLKQCFSLSD	
CD26	749	Q-I---S--I-----P	

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FIG. 2

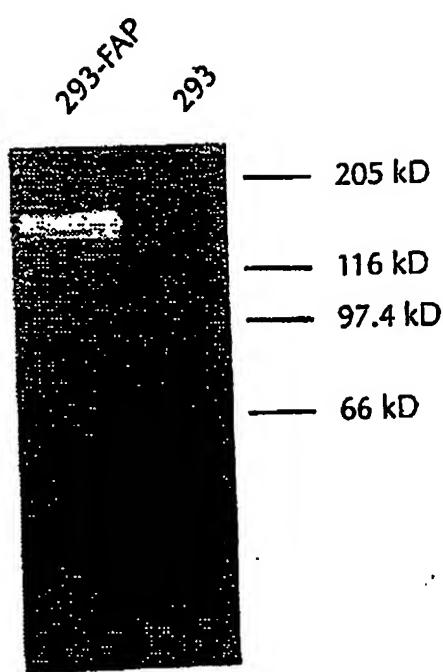
	Breast Cancer	MFH	Healing Wound	Renal Cancer
FAP $\alpha$	(+) A	(+) C	(+) E	(-) G
CD26	(-) B	(-) D	(+) F	(+) H

Immunohistochemistry (See Kodachromes)

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**FIG. 3**



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